UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Contribution for the knowledge on curable sexually transmitted infections with special emphasis on *Chlamydia trachomatis*

Zohra Gulzar Lodhia

Mestrado em Biologia Humana e Ambiente

Dissertação orientada por:
Doutora Maria José Borrego
Professora Doutora Deodália Dias

2017

Dedication

This dissertation is dedicated to the love of my life, the joy of my soul and the warmth of my heart, my baby niece *Mariam Shanawaz Lodhia*. The one that reassures my beliefs and hopes with just a glance from those amazing almond-shaped eyes and whose toothless smile makes my day, every day, every time.

Little hooman, my goduchas, *Jola Fui* loves you so much and will always be there for you, my beautiful princess. ♥

"You are my one and only,
you can wrap your fingers
round my thumb and hold
me tight…
And you'll be alright"

♥

For baby *Bilal*

Acknowledgements

First of all, I would like to thank Allah, to Whom I pray every single day and night, Who has been granting me so many blessings over the years, helping me throughout my struggles and giving me patience & strength.

A special thank you to my advisor, Dr. Maria José Borrego, responsible for the National Reference Laboratory for Sexually Transmitted Infections of INSA, for accepting me in her laboratory, for being such an amazing mentor who taught me so much, for her sympathy, thoughtfulness, patience, advices and for always being there when I needed.

To my internal advisor and masters' coordinator, Professor Dr. Deodália Dias, for always being available, for her kindness and for always figuring out a solution to every problem.

To Dr. Vítor Borges, researcher at the Bioinformatics Unit of the Infectious Diseases Department at INSA, for his contribution on my dissertation, for his teachings, gentleness and friendship.

To Miguel Pinto, my fellow lab colleague, who has the best playlists of the laboratory and with whom I learned so much on so many subjects (non-scientific included). Thank you for making me *'meow'* every single song in my head, for the productive conversations and discussions, for helping me in some aspects of my dissertation, for the friendship, kindness and all the laughter.

To Dr. João Paulo Gomes, responsible for Research Unit of the Infectious Diseases Department at INSA, for his sympathy, professionalism and sense of humor.

To Professor Dr. Marília Antunes, for finding some time in her busy agenda to help me with the statistical analysis of this dissertation.

To my amazing parents, for giving me the possibility to pursue another step in my academic formation, for always being comprehensive, for insisting so much with me when I did not feel like doing a break from my work to go to eat because I was so focused, for supporting, caring and loving so much their baby girl.

To my brother and sister-in-law who are both always so caring and crazy, but mostly supportive, for always wishing me the best, spoiling me with so much love and '*bussa bussi*' and for putting up with my snapchats when I was in my many *'fritanços'*.

To Dani (Daniela Ferreira), my best friend, the one that is always there when I need, for always helping me to see the sunny side of everything, for cheering me up, for caring and worrying, for reminding me what I am capable of and for always having the best future plans ever.

To my Madrinha Sissi (Sílvia Firmino) for being the best godmother I could have asked for, for always treating me as her *'piqui'*, for always sending me messages asking how things were going and for always reassuring I could count on her, no matter what.

To Abdullah & Munisa, thank you for insisting, calling me on my phone a hundred times and basically forcing me to go out to unwind when most of the times I did not even feel like it, but surely needed even when it was just for a 'quick coffee break'.

To Fátima Karolia, ‘mommy dearest’, the sister I got to “choose”, for all the support, all the crazy talks and laughs and for “motivational baby Adam” photos, videos and video-calls.

To Saf (Safwaan Zamakda), my cousin, who was always so nice and thoughtful and, although he is literally on the other side of the world, he was always so supportive, always cheering me up and rooting for me.

To Neves (André Lopes), one of the most amazing friends I have, for our lunch breaks, for our conversations and hilarious stories and for holding and helping me when my world was turned upside down and I felt like I could not reach the light and you led me right to it.

To all my friends: a HUGE thank you for all the support, for believing in me, cheering me up, giving me strength, caring, worrying, loving, making me laugh, for the many dinners out and for all the “you are going to make it, do not worry!”.

It was a really exhaustive and long time, fulfilled with so much hard work, so much knowledge and challenges and I could not be any more grateful than I am for all those who were part of this journey and for those who somehow helped me get through it.

THANK YOU, from the bottom of my heart. ♥

Resumo

Introdução e objetivos: As infeções sexualmente transmissíveis (ISTs) abrangem um amplo conjunto de síndromes com quadros clínicos variados que vão desde a ausência de sintomas até grave morbilidade, incluindo a própria morte. Existem mais de 30 agentes patogénicos de origem bacteriana, viral ou parasitária, que podem ser transmitidos de uma pessoa para outra durante o ato sexual. As ISTs estão entre as condições agudas mais comuns no mundo, sendo *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* e *Mycoplasma genitalium* as quatro principais causas de ISTs curáveis, sendo responsáveis por uretrite, cervicite, vaginite e proctite. Em Portugal, não há dados sobre a prevalência destas ISTs, desconhecendo-se o seu respetivo impacto para saúde pública. Deste modo, a presente dissertação teve por objetivo: **a)** o estudo da prevalência dos quatro microrganismos acima referidos no contexto de uma consulta de ISTs (Unidade de consulta de DST/CAD da Lapa); **b)** a análise da variabilidade genética do gene usado para genotipagem de *C. trachomatis*, *ompA*, o qual codifica a principal proteína da membrana externa de *C. trachomatis*, com vista a caracterizar as diferenças genéticas entre estirpes, as quais podem estar subjacentes a um processo contínuo de evolução e adaptação desta bactéria. Foram usadas as estirpes reunidas pelo laboratório de acolhimento ao longo de um período de 27 anos; **c)** o estudo da variabilidade intra-estirpes de *C. trachomatis*, de alvos potencialmente associados à variação de fase e à adaptação bacteriana no contexto da infeção *in vivo*. A variação de fase, que pode ser causada por mutações reversíveis que influenciam a expressão génica e a sua função, é conhecida por ser essencial para a adaptação e virulência de algumas bactérias, mas pouco se sabe sobre o seu eventual papel na biologia e na patogenicidade de *C. trachomatis*.

Materiais e Métodos: a) Foram avaliadas 1034 amostras biológicas colhidas na consulta DST/CAD Lapa, durante o período de tempo compreendido entre Setembro de 2016 e Setembro de 2017, relativamente à presença de *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis* e *M. genitalium*, utilizando dois sistemas de PCR em tempo real, Cobas® 4800 CT/NG (Roche Sistemas de Diagnóstico) e S-DiaMGTV™ (Diagenode); **b)** Foi efetuada genotipagem-*ompA* de 370 amostras utilizando uma técnica de nested-PCR, seguida de sequenciação pelo método de Sanger e a análise de similaridade das sequências genéticas obtidas relativamente à sequência *ompA* de estirpes protótipo de *C. trachomatis*. Posteriormente, essas amostras foram incluídas na base de dados do laboratório de acolhimento, num total de 2579 estirpes de *C. trachomatis* reunidas entre 1990 e 2017, tendo sido analisada toda a coleção relativamente ao genótipo *ompA*, ao género, à localização anatómica da infeção e à distribuição das variantes genotípicas; **c)** Foram analisadas 167 amostras (96 amostras de DNA selecionadas de entre as constantes na base de dados de *C. trachomatis* do laboratório, juntamente com um conjunto de 71 amostras para as quais os dados de sequenciação total do genoma tinham sido recentemente disponibilizados) relativamente a 12 tratos homopoliméricos potencialmente variáveis no genoma desta bactéria, através de uma técnica com base na sequenciação de nova geração (NGS), de produtos de amplificação (amplicões).

Resultados: a) Um quinto da população estudada estava infetada por pelo menos uma IST, tendo sido *C. trachomatis* a mais frequentemente detetada, seguida por *N. gonorrhoeae*, *M. genitalium* e *T. vaginalis*. Os indivíduos do sexo masculino revelaram-se mais frequentemente infetados que as mulheres, com especial ênfase para os indivíduos com idades compreendidas entre os 25 e os 34 anos, e para os indivíduos com mais de um parceiro sexual. No nosso estudo, *N. gonorrhoeae* foi a segunda IST mais frequente, sendo que os homens contribuíram com mais de dois terços dos casos e que cerca de metade deles eram homens que faziam sexo com homens (HSH); **b)** A genotipagem-*ompA* de estirpes clínicas de *C. trachomatis* evidenciou 12 genótipos-*ompA*, de entre os quais os genótipos E e F foram os mais frequentes, enquanto os genótipos B e o C foram os mais raros. Os genótipos G e L2 foram

muito mais comuns nos homens do que nas mulheres, tendo sido o endocervix/uretra o local anatómico onde mais frequentemente se efetuou a pesquisa e, consequentemente, se detetou a infeção. Aliás, o endocervix/uretra foi o único local anatómico onde os 12 genótipos foram detetados; **c)** Os resultados deste estudo revelaram diferentes perfis de variação intra- e inter-paciente para os poli (Ns) estudados, os quais inequivocamente sublinham o papel de um homopolímero em desencadear mecanismos reversíveis de "ON/OFF" da citotoxina de *C. trachomatis* (CT166) *in vivo*. Foram ainda identificados outros novos potenciais mediadores de mecanismos de variação de fase em *C. trachomatis*.

Discussão: a) De acordo com os dados de prevalência de ISTs nos EUA e na Europa, nos quais *C. trachomatis* é descrita como a IST bacteriana mais frequente, e com o aumento do número de casos que têm sido descritos nos últimos anos, era esperado que *C. trachomatis* fosse a IST mais frequentemente detetada na população estudada. No entanto, as taxas de prevalência podem subestimar a verdadeira dimensão desta infeção dado o seu caráter assintomático. Por outro lado, a sua maior frequência pode também ser apenas um reflexo da melhoria dos sistemas de vigilância das ISTs, em muitos países, muitas vezes suportados por estudos de rastreio em populações assintomáticas; tais estudos são facilitados pela disponibilidade de melhores ferramentas de diagnóstico laboratorial (sensibilidade e especificidade próximas dos 100%). O número de casos positivos de ISTs na região anorretal e na orofaringe, testadas apenas em HSH, justificam a necessidade de alargar o diagnóstico a esses locais anatómicos em homens heterossexuais e em mulheres. Deste modo, e considerando que este estudo envolveu pacientes de apenas uma consulta IST, é de salientar a necessidade de uma avaliação a nível nacional, uma vez que até à data a prevalência destas ISTs na população portuguesa é desconhecida; **b)** A elevada frequência dos genótipos-*ompA* E e F, e a menor ocorrência de estirpes variantes nas estirpes destes genótipos, sugere uma melhor adaptação desses genótipos; tal situação favorecerá que não sejam reconhecidos pelos mecanismos de defesa do hospedeiro e que não sejam eliminados pelos sistemas de defesa do hospedeiro, facilitando a sua disseminação e justificando o seu predomínio em termos epidemiológicos, em detrimento doutros genótipos-*ompA*. No entanto, são necessários estudos envolvendo o genoma total de estirpes clínicas de *C. trachomatis*, que deverão ser acompanhados pela análise dos respetivos dados clínicos, para uma mais completa compreensão das características genéticas de *C. trachomatis* e a sua relação com o desenvolvimento de patologia; **c)** Este estudo enriquece o conhecimento sobre a variabilidade intra-paciente de tratos homopoliméricos potencialmente mediadores de mecanismos de variação de fase, consolidando a hipótese de que a funcionalidade da conhecida citotoxina de *C. trachomatis* (CT166), durante o processo infecioso, será regulada por mecanismos de variação de fase, o que ficou evidenciado pela elevada variabilidade inter e intra-paciente. Por outro lado, foi também possível demonstrar, pela primeira vez, que existem outros tratos homopoliméricos com variabilidade intra-estirpe para além do referido acima. É de destacar a necessidade de se alargar esta abordagem, utilizando-se um conjunto mais homogéneo e amplo de amostras de *C. trachomatis*, considerando também os respetivos dados clínicos e os genes relacionados putativamente com mecanismos de variação de fase, de modo a consolidar algumas das hipóteses suscitadas no decurso da presente dissertação de mestrado.

Palavras-chave: Infeções sexualmente transmissíveis curáveis; *C. trachomatis*; Genótipos-*ompA*; Variação de fase; Homopolímeros.

Abstract

Introduction and objectives: Sexually transmitted infections (STIs) refer to a wide variety of clinical syndromes and infections, which are among the most common acute conditions in the world, causing serious morbidity, and even death. *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Trichomonas vaginalis* and *Mycoplasma genitalium* are the four main causes of curable STIs, being responsible for urethritis, cervicitis, vaginoses and proctitis. In Portugal, there is no data on their prevalence, being unknown the impact of these STIs. This dissertation aimed at **a)** studying the prevalence of these four microorganisms in the context of a sexually transmitted diseases (STDs) consultation; **b)** analyzing the variability of *C. trachomatis* genotyping gene, *ompA* – which codes to the major outer membrane protein –, throughout a timeline of 27 years. The genetic differences detected in this gene may provide important clues about the continuous process of evolution and adaptation of this human pathogen; **c)** studying *C. trachomatis* intra-strain variability *in vivo*, as a means to identify potentially phase-variable targets associated with bacterial adaptation in the context of infection.

Materials and Methods: **a)** We evaluated 1034 samples collected from patients of the major Portuguese STD clinic (Unidade de consulta de DST/CAD da Lapa) for the microorganism's presence using two real-time PCR systems, Cobas® 4800 CT/NG (Roche Sistemas de Diagnóstico) and S-DiaMGTV™ kit (Diagenode); **b)** *ompA*-genotyping of 370 samples was performed using a nested-PCR technique, Sanger sequencing and *ompA*-sequence similarity analysis regarding *C. trachomatis* prototype strains. Then, we added these samples to the Instituto Nacional de Saúde Doutor Ricardo Jorge database collection, composed by 2579 *C. trachomatis* specimens, gathered from 1990 to 2017, and analyzed the whole database regarding *ompA*-genotype, gender, anatomical site of infection and genotypic variants distribution; **c)** We analyzed 167 samples (96 selected *C. trachomatis*-positive DNA samples from the laboratory database, along with a set of 71 samples for which whole-genome sequencing data had been recently released) with an amplicon-based next generation sequencing (NGS) technique for 12 selected potentially variable poly (Ns).

Results: **a)** We found that almost a fifth of our study population was infected by at least one STI, with *C. trachomatis* being the most commonly detected, followed by *N. gonorrhoeae*, *M. genitalium* and, finally, *T. vaginalis*. Men were, in general, more infected than women, with especial evidence for individuals aged 25 to 34 years, and for people who had more than one sexual partner; **b)** *ompA*-genotyping of *C. trachomatis* clinical strains evidenced 12 *ompA*-genotypes, among which E and F were the most represented and B and C were the least frequent. Genotypes G and L2 were much more common among men than among women and the endocervix/urethra was the anatomical site of infection more represented, and the only site where all genotypes could be detected; **c)** The scrutiny of the selected potentially variable poly (Ns) revealed distinct trends of inter- and intra-patient variation that, not only unequivocally reinforce the role of a reversible poly(N) in the ON/OFF switching of *C. trachomatis* cytotoxin (CT166) *in vivo*, but also launch other poly(N)s as potential mediators of phase variation mechanisms in *C. trachomatis*.

Discussion: **a)** According to its prevalence in the USA and Europe, and because the number of detected cases have been rising the past few years, *C. trachomatis* was expected to be the most frequently detected STI. However, reported prevalence rates underestimate the true burden of this STI due to its asymptomatic character. On the other hand, its frequency may also reflect the expansion of screening, namely among asymptomatic because of the development of better and more sensitive diagnostic tools, and also due to the improvement of reporting systems. The rectum and the oropharynx were only tested in MSM, and the number of positive STIs cases in these anatomical locations highlight the need for their investigation among heterosexual men and women; **b)** The high frequency of E and F together with the low occurrence of variant strains suggest a better fitness of these *ompA*-genotypes that would favor them to go undetected by the host defense mechanisms, facilitating their dissemination, and consequently leading to their predominant epidemiological rates. However, genomic studies involving the whole genome sequence together with clinical data are required for a more complete understanding of the genetic features of *C. trachomatis*; **c)** This study constitutes an unequivocal turning point on our knowledge of the intra- and inter-patient heterogeneity affecting *C. trachomatis* poly (Ns), by identifying novel potential targets of phase variation in the context of *C. trachomatis* infection. In particular, it consolidates the hypothesis that the functionality of the well-known *C. trachomatis* cytotoxin (CT166) is regulated by an ON/OFF mechanism of phase variation during infection. A future scale-up of the strategy applied in the present study approach, using an homogeneous and wider range of *C. trachomatis*-positive DNA samples together with their clinical background, along with other putative phase variation related genes, should be performed in order to consolidate some of the hypothesis raised through the present master's thesis.

Key-words: Curable sexually transmitted infections; *Chlamydia trachomatis; ompA*-genotypes; Phase variation; Homopolymers.

Table of Contents

Figure Index

Table Index

List of abbreviations, acronyms and symbols

µL – Microliter;

µm – Micrometer;

AB – Abnormal Body;

AMR – Anti-microbial resistance;

BV – Bacterial Vaginosis;

CAD – Centro de Aconselhamento e Deteção (portuguese);

CD – Conserved Domain;

CDC – Centers for Disease Control and Prevention;

COMC – Chlamydia Outer Membrane Complex;

CT – *Chlamydia trachomatis*;

DFA – Direct immunofluorescence assay;

DNA – Deoxyribonucleic acid;

DST – Doença sexualmente transmissível (portuguese);

EB – Elementary Body;

ECDC – European Centre for Disease Prevention and Control;

ELISA – Enzyme-linked immunosorbent assay;

EUA – Estados Unidos da América (portuguese);

EU/EEA – European Union and the European Economic Area;

FCUL –Faculty of Sciences, University of Lisbon (from the Portuguese Faculdade de Ciências da Universidade de Lisboa);

HIV – Human Immunodeficiency Virus;

HSH – Homens que fazem sexo com homens (portuguese);

HSV – Herpes Simplex Virus;

IFN-γ - Interferon-γ;

INSA – National Institute of Health (from the Portuguese Instituto Nacional de Saúde);

IST – Infeção Sexualmente Transmissível (portuguese);

LGV – Lymphogranuloma venereum;

LPS – Lipopolysaccharide;

MACPF – Membrane Attack Complex/Perforin;

MOMP – Major Outer Membrane Protein;

MG – *Mycoplasma genitalium*;

MSM – Men who have Sex with Men;

NAAT – Nucleic acid amplification test;

NAH – Non-amplified nucleic acid hybridization assays;

NG – *Neisseria gonorrhoeae*;

NGS – Next-Generation Sequencing;

NGU – Non-Gonococcal Urethritis;

ORF – Open Reading Frame;

PBS - Phosphate-buffered saline;

PCR – Polymerase Chain Reaction;

PID – Pelvic inflammatory disease;

PLD – Phospholipase D;

PMP – Polymorphic Membranes Proteins;

POC – Point-of-care;

PROM – Premature Rupture of Membranes;

PZ – Plasticity Zone;

RB – Reticulate Body;

RFLP – Restriction Fragment Length Polymorphism;

RNA – Ribonucleic acid;

SARA – Sexually acquired reactive arthritis;

STD – Sexually Transmitted Disease;

STI – Sexually Transmitted Infection;

T3SS – Type III Secretion System;

TARP – Translocated Actin Recruiting Phosphoprotein;

TFI – Tubal Factor Infertility;

TOC – Test-of-cure;

TV – *Trichomonas vaginalis*;

UDF – User-defined workflow;

UK – United Kingdom;

USA – United States of America;

USPSTF – United States Preventive Services Task-Force;

VD – Variable Domain;

WHO – World Health Organization;

XDR – Extensive drug resistance.

Introduction

1. Sexually Transmitted Infections

The term “Sexually Transmitted Infections” (STIs) refers to a wide variety of clinical syndromes and infections, which are among the most common acute conditions in the world [1]. There are more than 30 bacterial, viral, and parasitic pathogens [2, 3] that can be transmitted from one person to another person during sexual intercourse [2, 4].

The World Health Organization (WHO) estimates that more than 1 million STIs are acquired everyday worldwide, and that 357 million new episodes of four STIs, *Chlamydia trachomatis* (131 million), *Neisseria gonorrhoeae* (78 million), *Treponema pallidum* (6 million) and *Trichomonas vaginalis* (142 million) occur every year [5].

About 20 million STIs cases are annually reported to the Centers for Disease Control and Prevention (CDC) of the United States, which are considered an epidemic of tremendous magnitude and a public health concern [6].

Adolescents and young adults aged between 15 to 24 years old account for the majority of STIs cases because of a combination of biological, behavioral and cultural reasons; moreover, the earlier people start their sexual life, the higher are the chances of acquiring a STI [6, 7]. Some populations are at higher risk of getting a STI, i.e. men who have sex with men (MSM), intravenous drug users, people who have multiple partners, and non-users of condoms [6, 8, 9, 10].

The most predominant STIs symptoms are the urogenital infections, which are often associated with urethritis and epididymitis in men and cervicitis and pelvic inflammatory disease [11]. However, because most of these infections are frequently asymptomatic, people do not realize they are infected [12, 13] and accordingly, fail to seek treatment, which may lead to severe complications, long term sequelae, and a variety of diseases across multiple organ systems. If not treated, some STIs may cause ectopic pregnancy or tubal infertility. Some STIs can also be vertically transmitted, from an infected mother to her infant, inducing serious consequences for the offspring, such as neonatal death, premature delivery, blindness or severe disability in infants [1, 3, 11], justifying the need for screening every pregnant woman. However, STIs are frequently associated with social stigma, social stereotyping and vulnerability, and shame, which justify the trying of hiding a (potential) condition and the avoidance of screening (and treating) these infections.

Low incidence rates often reflect differences in healthcare systems, lack of accurate diagnostic tools, or insufficient diagnostic capacity, rather than a genuinely low or declining frequency of these infections. These reasons also lead to the underestimation of the true prevalence of STIs [9]. Therefore, increasing the scope and accessibility for screening STIs would provide an opportunity to identify and treat these infections, a public health priority. Laboratory and point-of-care (POC) tests are potentially powerful contributors to the management and control of STIs, facilitating prevention by precluding transmission. Although there are a wide variety of tests that can be applied to diagnose STIs, the CDC recommends the use of nucleic acid amplifications tests, also known as NAATs [2, 14].

The present Master's thesis focuses on curable STIs main agents, *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis* and *Mycoplasma genitalium,* which are responsible for urethritis, cervicitis, vaginitis and proctitis, and related clinical sequelae.

2. *Chlamydia trachomatis*

Chlamydia trachomatis is an obligate intracellular gram-negative bacterium and it belongs to the genus Chlamydia (phylum Chlamydiae, order Chlamydiales, family Chlamydiaceae) [15].

2.1. The life cycle

Throughout an evolutionary process undertaking several millions of years, Chlamydiae evolved developing mechanisms for interacting and colonizing eukaryotic host cells (usually epithelial) in which they go through a biphasic developmental cycle, unique in nature. During this life cycle with duration of 48 – 72h, the bacterium alternates between two highly specialized morphological forms: the elementary bodies (EBs) and the reticulate bodies (RBs) [16].

The metabolically inert infectious EBs are small (~ 0,3μm), have a coccoid form and the external membrane contains extensive disulfide cross-links, within and between outer membrane proteins, providing it with a "sporelike" structure, i.e. a rigid cell wall that keeps the microorganism stable outside the cell, allowing its survival in the extracellular environment for a short period. On the other hand, RBs are larger (~ 1 μm), noninfectious, metabolically active, structurally flexible and osmotically fragile, richer in RNA and containing diffuse and fibrillar DNA [14, 17].

The chlamydial infectious process may be divided into five major events (**Figure 1.1**). It begins with the attachment and entry of the EB into the host epithelial cell, triggering host actin reorganization, membrane deformation, and internalization as endocytic vacuoles [17, 18, 19]. Subsequently, the EB differentiates into RB inside the inclusion, and gene transcription starts [20]. The inclusion suffers some modifications, and the RB replication occurs by polarized cell division processes [21]. Next steps are inclusion expansion and RB transition into EB. Finally, EBs are released by host cell lysis or extrusion, a phenomenon that occurs 30 – 72h post-infection (depending on species and strain) and that allows them to attach to neighboring epithelial cells and to initiate a new cycle [16, 20, 22, 23, 24].

However, the developmental cycle might be interrupted under stressful conditions caused by host immunological response, nutrient starvation or antibiotic treatment and, until these stress factors are resolved, EBs convert into noninfectious, enlarged, aberrant bodies (ABs) [25].

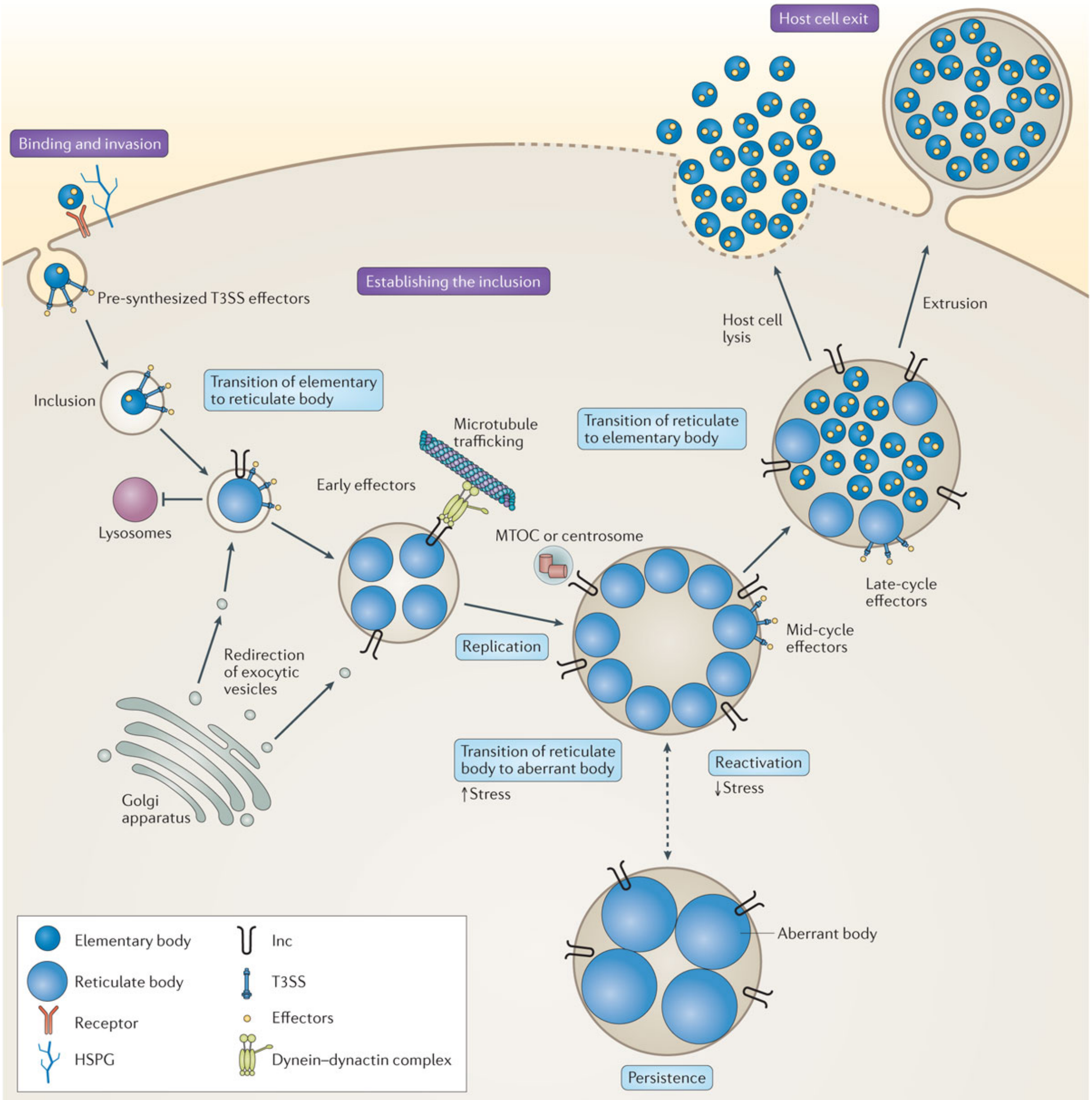


Figure 1.1 – Schematic representation of the development cycle of *C. trachomatis* [20].

2.2. Biological and genomic features

As any gram-negative bacteria, *C. trachomatis* is provided with a double membrane. The outer membrane is constituted by the chlamydia outer membrane complex (COMC) which includes the major outer membrane protein (MOMP), which accounts for 60% of the membrane dry weight, OmcA, OmcB, PorB, OprB, and OmcB, the type III secretion system (T3SS), the polymorphic membranes proteins (Pmps), porins, lipids and polysaccharides (LPS) [26, 27, 28, 29].

COMC proteins are the first to interact with the host during infection and they maintain the integrity of the chlamydial infectious particle [29]. In fact, MOMP is the major chlamydial antigen and a serologic type classification could be established based on it**;** during chlamydial replication, MOMP may act as a porin and also contributes to the structural integrity of the cell [26, 27]. The majority of the MOMP amino acid sequence is conserved, but it contains four symmetrically spaced variable domains (VDs) – VDI:

residues 64 to 83; VDII: residues 139 to 160; VDIII: residues 224 to 237; and VDIV: residues 288 to 317**,** flanked and interspaced by five conserved domains (CDs) [30, 31]. The antigenic determinants of MOMP allowed to elicit 15 serotypes/serovars in *C. trachomatis*, A to L3, and serotype-specific monoclonal antibodies were developed to recognize them [11, 32]. The ocular biovars (serovars A, B, Ba, and C) origin trachoma, a conjunctival infection that can lead to blindness and constitutes the most common cause of preventable blindness in the world, being endemic in many developing countries [2, 24]. The urogenital biovars (serovars D-K) are sexually transmitted, where E is the most common followed D and F; serovars D, G, and J tend to predominate among MSM [10, 32, 33, 34, 35, 36, 37]. Serovars D to K can cause ocular infections in neonates and adults, by secondarily inoculation of the eye with infected genital secretions [32]. The LGV biovar (L1, L2, and L3) is also sexually transmitted, but the infection often spreads to the regional draining lymph nodes and to the rectum causing lymphadenitis and proctitis [2, 17, 38].

With the advent of molecular biology techniques, the *ompA* gene that codes for MOMP evidenced dissimilarities that allowed to differentiate 15 genotypes [39]. Genotyping became the methodology of choice to distinguish the particular *C. trachomatis* types and variants involved in each infection, which is an important tool to understand *C. trachomatis* epidemiology and pathogenesis; in fact, correlating *ompA* genotypes and clinical manifestations, could help to develop strategies for disease control, such as vaccines. Genotyping would also be useful to differentiate between persistent and new infections, to identify outbreaks and transmission patterns among sexual networks, and would allow the surveillance of specific types of interest [37, 40, 41]. *ompA*-genotyping subdivides *C. trachomatis* strains into three distinct phylogenetic clades, the B-complex (B/Ba, D, E, L1, and L2), the C-complex (A, C, H, I, J, K, and L3), and the intermediate complex (F and G) [11]. However, there is no correlation between these phylogenetic clades and disease or tissue tropism [42].

The genome of obligate intracellular parasites is known to be relatively small when compared to free-living bacteria. While becoming metabolic parasites, they went through a lot of biological processes, in particular genome reduction, as they will benefit from living in the host cytoplasm, where they can obtain high-energy phosphate compounds [17, 43].

C. trachomatis is also thought to have undergone this genome reduction pathway, likely from nonpathogenic ancestors that became specialized to infect humans, while maintaining the ability to infect different tissue types, and cause human diseases of major public health significance [44, 45, 46]. It comprises a single circular chromosome with approximately 1 Mb and ~7 kb plasmid, with a highly conserved genome, with the exception of the plasticity zone (PZ), near the replication terminus, that is dissimilar among *C. trachomatis* strains [14, 47, 48, 49]. Genes in the PZ include the tryptophan synthase (*trp*), the cytotoxin, the membrane attack complex/perforin (MACPF), and the phospholipase D (PLD), and they enclose strain- and species-variable alleles [50].

The *trp* operon is a virulence factor, which in the presence of the host interferon IFN-γ is able to synthesize tryptophan [39]. However, only the genital genotypes exhibit an intact operon encoding a functional tryptophan synthase, whereas ocular strains display mutations in the *trpA* or *trpB* genes that result in a nonfunctional synthase, providing a metabolic distinction between strains that is nearly axiomatic with tissue-specific appetence [51, 52].

The chlamydial cytotoxin is a highly polymorphic gene that is thought to act on the rapid disassembly of the cytoskeleton actin filaments during the bacterial internalization process, but only the genital genotypes encode both the functional glycosiltransferase and the UDP-glucose binding domains of the cytotoxin open reading frame (ORF), CT166, while ocular genotypes encode only the UDP-glucose binding domain, and LGV genotypes lack the cytotoxin ORF [14, 53].

On the other hand, genes like MACPF and PLD are present in all genotypes, taking part in the processes of acquisition of metabolites from the host, which are essential for the survival and success of the developmental cycle [50].

There are genes outside the PZ that are related to virulence and pathogenicity, such as the *Pmp* genes, TARP (translocated actin recruiting phosphoprotein) genes, *Inc* genes and *ompA* [40]. The T3SS effector TARP is spatially and temporally associated with the rapid polymerization of actin filaments required at the site of EB invasion, with a high degree of variability [54]. *C. trachomatis* uses it to translocate virulence effector proteins directly into the host cells cytoplasm, where they subvert cellular processes to promote cell invasion, inhibition of phagocytosis, establishment of the inclusion, acquisition of nutrients, modulation of intracellular trafficking, early inhibition, late induction of apoptosis, and avoidance of innate immune responses, all contributing for chlamydial pathogenesis. [55, 56, 57]. *Inc* genes code for proteins (Inc's) that are inserted in the inclusion membrane, exposed to the host cell cytosol, and are also effectors of the T3SS family.

Pmps are coded by a highly heterogenic gene family with a different number of *pmp* genes intra and interspecies, which have been characterized as adhesins and autotransporters [40, 45, 58].

LPS are the main lipid present in the outer membrane, functioning as a permeability barrier that avoids bacterial cell-damaging agents such as detergents, proteases, bile salts, and hydrophobic antimicrobials, being essential to *Chlamydia sp*. [59].

Due to the genus, species and type epitopes present in MOMP, inducing both humoral and cellular immune response in the host, its encoding gene, *ompA*, is provided with variability and antigenic dissimilarities capable of constituting *ompA* as a good candidate for the development of a vaccine [41].

Phase variation mechanisms are known to play an important role in bacterial adaptation and pathogenesis, relying on the ability of bacteria to rapidly adapt in response to stimuli, usually associated with a reversible switching between ON and OFF state of specific proteins, such as proteins involved in biosynthesis/expression of the bacterial capsule and LPS [60, 61]. These mechanisms normally occur due to expansion/contraction of genomic homopolymeric tracts, described as repetitive regions of DNA single base, leading to different phenotypes [62, 63, 64]. Although it is still not well-established if *C. trachomatis* employs these mechanisms to promote adaptation and virulence, this subject warrants further investigation as recent findings have revealed intra-strain heterogeneity targeting homopolymeric tracts with potential to shape *C. trachomatis* virulence [60].

2.3 Epidemiology and pathology

C. trachomatis is the most common bacterial STI, infecting about 131 million people, worldwide, each year [1]. The number of cases has grown during the last decade; in fact, the CDC reports an increase of 2.8% in 2014 (1 441 789 cases reported in the United States), in relation to 2013 and the ECDC reported an increase of 67% from 2004 to 2013 among consistently reporting countries [7, 65, 66]. However, this rise may be a consequence of the widespread testing of asymptomatic individuals, and of the introduction of diagnostic tests with higher sensitivity [7, 10, 13, 43, 48, 67, 68, 69].

C. trachomatis prevalence is usually higher in people younger than 24 years [4, 17, 69]. In Europe, in 2013, two thirds of the *C. trachomatis* cases were reported in young adults aged between 15 to 24 years, affecting both men and women, although more frequently reported in women, and were mostly (88%) heterosexually transmitted [7, 43, 66, 67]. Multiple/high number of sexual partners, having a sexual partner with a STI, inconsistent condom use, and/or have another STI, are some of the risk factors for getting a *C. trachomatis* infection [4, 12].

Despite the estimations described above, *C. trachomatis* true incidence and prevalence are likely to be significantly higher; in fact, the asymptomatic nature – which allows the infection to go unnoticed until more severe symptoms develop – and the differences in testing methods, testing coverage, screening programs, and surveillance systems, many cases might be neither diagnosed nor reported. In fact, 83% of the data available for Europe are based in four countries – Denmark, Norway, Sweden and the United Kingdom – the countries where *C. trachomatis* reporting seem most effective [66]. Thus, a large burden of disease is expected and the health associated risks justify the CDC recommendation for annually screen all sexually active women under 25 years, as well as older women at high risk of infection, to prevent sequelae, and to test and treat their sexual partners [4, 7].

According to the USPSTF (United States Preventive Services Task-Force) [70], there is insufficient evidence to recommend routine *C. trachomatis* screening in sexually active young men, because of several factors (e.g., feasibility, efficacy, and cost-effectiveness); however, the screening of sexually active young men should be considered in clinical settings where a high prevalence of *C. trachomatis* is to be expected (e.g., adolescent clinics, correctional facilities, STD clinics, and MSM).

Although 70 – 95% of the *C. trachomatis* infected women are asymptomatic, symptoms might occur which include urethritis, dysuria, vaginal discharge, postcoital bleeding, cervicitis, mucopurulent cervical discharge, cervical friability, cervical edema, endocervical ulcers, mid-cycle spotting, poorly differentiated abdominal pain or lower abdominal pain and proctitis [71]. If left untreated, *C. trachomatis* infection can progress and damage the upper reproductive tract, leading to pelvic inflammatory disease (PID), tubal factor infertility (TFI), chronic pelvic pain, ectopic pregnancy, endometritis, salpingitis or sexually acquired reactive arthritis (SARA) [4, 7, 10, 17, 43, 67, 71, 72, 73]. Repeated *C. trachomatis* infections might increase the risk of PID and tubal damage [12]; however, there has been a decline in the hospitalization rates for PID and ectopic pregnancy, since the introduction of *C. trachomatis* control programs, in women [67, 74, 75].

In men, more than 50% of the *C. trachomatis* infections are asymptomatic; when present, symptoms might appear as penile tip irritation, watery and viscous excretions, urethral discharge, proctitis and epididymitis. *C. trachomatis* seems to neither exerts deleterious effects on spermatozoa nor impairs male fertility, unlike to the described for women [76].

It is assumed that 20 – 54% of the uncomplicated urogenital infections will clear spontaneously in 1 year, in average [24, 77].

For both genders, the clinical signs and symptoms are not exclusive of the *C. trachomatis* infection as they can be caused by other STIs like *T. vaginalis, N. gonorrhoeae* or *M. genitalium* (common STIs coinfections) and candidiasis and a laboratory diagnosis is required for confirming the case [2, 7, 35, 38, 67, 71, 78]. Moreover, everyone who receives a diagnosis of *C. trachomatis* should be tested for HIV and syphilis [4].

Since 2004, in particular among MSM, outbreaks of lymphogranuloma venereum (LGV), an invasive infection, have been reported. LGV is caused by *C. trachomatis* L1, L2 and L3 strains, among which the L2b variant was referred as the most frequent [79, 80, 81, 82]. The main site of infection is the rectum and, although it might be asymptomatic, major symptoms include tenesmus, constipation, anorectal pain, mucopurulent discharge, bleeding per rectum, diarrhea, abdominal pain and proctocolitis (often confused with inflammatory bowel disease) [68, 71, 83, 84]. If not treated early, it can progress to chronic colorectal fistulas and strictures, lymphatic obstruction and, uncommonly, meningoencephalitis, hepatitis and death [85, 86].

Rectal chlamydial infections in women might become more frequent, as data from the UK shows that 15% - 17% of heterosexual couples reported anal sex practices [68]; however, no LGV outbreaks have been reported. Moreover, no association could be established between patients reporting anal intercourse and rectal chlamydia infection, suggesting that the infection could be caused by dispersion from the genital tract to the rectal site [24].

Pharyngeal chlamydial infections can also occur and its detection rates in MSM range from 0.5% to 2.3% [87, 88]. They are usually asymptomatic, but symptoms like mild sore throat can occur [89].

In women, untreated maternal chlamydial infections have been associated with premature rupture of membranes (PROM), preterm delivery, perinatal mortality and postpartum endometritis in addition to neonatal morbidity [13]. Neonatal infections are acquired during birth, when the baby passes through a *C. trachomatis* infected birth canal (risk range from 50% to 75%) [71, 90]. Infants may develop conjunctivitis – occurring in the first 3 weeks of life, where *C. trachomatis* replicates extensively in epithelial conjunctival cells, causing considerable cell damage – and/or pneumonia, being the nasopharynx the most frequent site of perinatally acquired *C. trachomatis* infection, with approximately 70% of infected infants having positive cultures at that site, occurring within the first 3 months of life [17, 38, 67, 71, 91]. Neonatal *C. trachomatis* infections may remain asymptomatic and persistence for as many as 3 years has been demonstrated; this hypothesis may cause confusion with *C. trachomatis* acquisition because of child sexual abuse [17]. An effective measure to prevent the transmission of mothers' *C. trachomatis* infection to the newborn would be screening and treating before delivery, which would highly reduce the risk of future complications [12, 17, 91].

For urogenital *C. trachomatis* infections (even for pregnant), azithromycin, 1g single dose, is considered the first-choice treatment; single-dose therapies tend to maximize adherence to treatment. Alternative treatment is doxycycline, 100mg two times daily for seven days. For LGV, because of its invasiveness, the recommended treatment is 100mg of doxycycline, orally, twice a day for 21 days [4, 67, 71]. To minimize the risk of transmission to sex partners, sexual intercourse abstention for 7 days after single-dose therapy or until completion of the regimen and resolution of symptoms, is required [4, 85, 92]. Test of cure (3 – 4 weeks after completion of therapy to avoid the presence of nonviable organisms that can lead to false-positive results) are not recommended, except for pregnant, as antibiotic resistance is considered rare; the fact that *C. trachomatis* is an intracellular parasite living inside a cell vacuole largely precludes exchanges of antimicrobial resistance genetic material [13]. Thus, most post-treatment infections do not result from treatment failure, but rather from reinfection with an untreated sex partner [4, 43, 71].

2.4 Diagnosis

C. trachomatis urogenital infection can be diagnosed in women by testing first-catch urine and endocervix (or vagina) swab specimens. In men, *C. trachomatis* urethral infection can be diagnosed by testing urethral swabs or, first option, first-catch urines [4, 38, 93]. However, anatomic sampling sites vary, according to sexual practices (anorectal and oropharynx samples might be required) and clinical presentation (conjunctivitis, arthritis, etc).

Bacterial isolation, in tissue culture, standardized in the 1970s, is no longer used for *C. trachomatis* diagnosis because it depended on maintaining organisms viable. This process required strict transportation (4ºC, 24h maximum) and laboratory storage (-70ºC or less) conditions which allied to the time and associated cost of cell culture justified the abandon of this method for diagnosis routine purposes [2, 32].

In the early 1980s, new techniques were developed, such as antigen detection assays, direct immunofluorescence assays (DFAs), and solid phase enzyme-linked immunosorbent assays (ELISAs) for a faster and easier detection of chlamydial particles in urogenital exudates. However, these methods lacked sensitivity, even when compared to culture and, most of all, suboptimal specificity, which justified not recommending them for *C. trachomatis* diagnosis [2].

The major advancement for *C. trachomatis* diagnosis was the employment of nucleic acid amplification methods. Initially there way non-amplified nucleic acid hybridization assay tests with a sensitivity similar to culture, but they were replaced by nucleic acids amplification tests (NAATs) that revolutionized *C. trachomatis* screening. NAATS employ enzymatic methods to exponentially amplify DNA (or RNA) targets into billions of copies and use sequence-specific probes with binding dyes for the detection of amplified DNA products, and become the methodology of choice for *C. trachomatis* diagnosis nowadays, combining specificity and sensitivity near to 100%. NAATS made possible to study a broader range of biological samples (including extragenital), less invasive and/or self-collected (i.e. urine, vulvar exudates) [2, 4, 38].

When using NAATs *C. trachomatis* viability is no longer a requirement, transport conditions are less strict, accommodating some delay between collection and laboratory processing without significant loss of sensitivity (nor specificity) [38]. Sample pooling is a possibility in resource-limited settings, and most of the commercially available tests were designed for detecting both *C. trachomatis* and *N. gonorrhoeae* simultaneously [2]. Additionally, NAATs are well suited to automation, which results in increased standardization and quality assurance of nucleic acid extraction, amplification and detection, as well as significantly increased throughput. Therefore, these tests became considered to have superior performance characteristics compared to any other test for the detection of *C. trachomatis* infections and, as such, they are the assay type recommended by the WHO and the CDC for both diagnosis and screening [2, 4].

3. *Neisseria gonorrhoeae*

The genus *Neisseria* contains two species primarily pathogenic to humans, *N. gonorrhoeae* and *N. meningitidis*, and approximately 30 usually nonpathogenic species such as *N. lactamica*, *N. sicca*, *N. cinerea*, *N. flavescens*, *N. subflava*, and *N. mucosa*. These organisms mainly inhabit the upper respiratory tract as commensals, but they can be found, infrequently, in the lower urogenital tract [2].

3.1. Epidemiology and human diseases

Gonorrhea is one of the earliest known human diseases with biblical references dating back to the Old Testament [94]. The causative agent of this infection, *N. gonorrhoeae*, is a gram-negative diplococcus, aerobic, non-flagellated, non-sporulating and it is a fastidious organism that requires complex nutritionally enriched culture medium for *in vitro* growth [2, 94, 95]. It has a marked tropism for human mucosal surfaces, affecting only humans, being transmitted almost exclusively through sexual contact [2].

N. gonorrhoeae is the second most common bacterial STI, after chlamydial infection [4, 7, 13, 96]. The ECDC refers an increase of 31% of reported cases between the years of 2008 and 2011, among the 28 EU/EEA Member States; in the year 2013, 52 995 cases were reported, with an overall rate of 17 cases per 100 000 population [66]. Between 2013 and 2014, the CDC describes an increase of 5.1%, with 350 062 cases of gonorrhea reported in the US only, and a rate of 110.7 cases per 100 000 population [7]. In most EU/EEA Member States, in 2013, about half of all gonorrhea cases (43%) were reported in MSM; in fact, since 2008, the overall rate has raised by 79%, mostly because of the increasing number of cases in men, especially MSM, a population recommended for screening by the ECDC at all anatomical exposure sites [66, 97].

In Portugal, the incidence of *N. gonorrhoeae* infection was estimated from 0.27 to 0.7 cases per 100 000 population but, because it is estimated that many cases remain undiagnosed or are not reported, the true burden of disease is likely higher [95].

N. gonorrhoeae prevalence is higher among adolescents and young adults, with ages from 15 to 24 years, accounting for 39% of all gonorrhea cases, in particular among those with a new sex partner, multiple sex partners, inconsistent condom use, having a sex partner with concurrent partners, or having a sex partner with a STI [4, 7, 66]. In contrast to chlamydial infections, gonorrhea is reported three times more often in men than in women, with 28.9 notifications per 100 000 in men in contrast to 9.7 per 100 000 in women; this might be explained by the increasing number of cases among MSM [7, 66].

After exposure, typical incubation period for men varies between two to five days [95, 98]. Gonococcal infections tend to cause a stronger inflammatory response than *C. trachomatis* but may remain asymptomatic until the development of complications [13]. The most common clinical presentations include acute urethritis, urethral discharge and dysuria; more rare complications may include penile edema, penile lymphangitis, periurethral abscess, acute and chronic prostatitis, seminal vesiculitis, urethral strictures and fistulae. Up to 60 – 80% of male patients have minimal or no symptoms at all [99, 100, 101].

For women, typical incubation period is a bit longer, varying from five to ten days following exposure, and most of them (>85%) are asymptomatic. When symptoms occur, the most common manifestations are cervicitis (with vaginal discharge), cervical bleeding, pruritis, and dysuria [98]. If left untreated, PID is the most common complication (10 – 20%), and often it presents as lower abdominal or pelvic pain. However, PID complications include endometritis, salpingitis, tubo-ovarian abscess, peritoneal and pelvic adhesions and, rarely, abdominal peritonitis or perihepatitis; infertility develops in approximately 15% of women with gonorrhea [94, 99, 100, 101, 102]. During pregnancy, gonorrhea may cause complications, such as chorioamnionitis, premature rupture of membranes, preterm birth and spontaneous abortions [103, 104].

Infections of the rectum and the pharynx are predominantly found in MSM, but, according to the sexual behaviors adopted, it can be found in both genders [2, 94]. Rectal infections are largely asymptomatic but, occasionally, patients may complain of rectal and anal pain or discharge. Pharyngeal infections are mainly asymptomatic too, but mild sore throat and pharyngitis may occur [2]. Ocular infections caused by *N. gonorrhoeae* are most commonly detected in neonates (ophthalmia neonatorum) and are acquired upon the passage through the birth canal of infected mothers. In adults, conjunctivitis by *N. gonorrhoeae* is due to auto-inoculation, and if it is left untreated, can lead to scarring and blindness [94, 98].

Repeated gonococcal infections increase the duration of erosion, the presence of local immune target cells, and infectivity; by altering host immune defenses, there is an increased risk of both acquisition and transmission of HIV [105, 106]. In fact, the risk of acquiring an HIV infection increases 8-fold for MSM who had two prior rectal *C. trachomatis* or *N. gonorrhoeae* infections [105].

When not treated, gonorrhea may evolve to complications such as meningitis and endocarditis. As described for *C. trachomatis*, every *N. gonorrhoeae* patient should also be tested for other STIs [4].

N. gonorrhoeae diagnosis is established by direct detection (microscopy of stained smears), culture or molecular biology techniques. In women, primary collection site is the endocervical canal or the vagina; in heterosexual men, specimens should be collected from the urethra. In the case of oral and/or anal sex, the rectum and the oropharynx should be sampled for testing [107].

Microscope observation of smears allow to identify gonococci as extracellular and, very often, intracellular diplococci in polymorphonuclear leukocytes. This is a cheap method, providing rapid results in symptomatic men with urethral discharge, with high sensitivity (95%) and specificity (97%) [2, 96]. In women, the same methodology can only detect 40 – 60% of the cases, which may reflect a lower number of gonococci in women cervical infections. Beyond the required experience of the laboratory technician, this methodology has a low sensitivity and cannot be used in the case of asymptomatic men, nor for pharyngeal or rectal gonorrhea, where commensal *Neisseria* species exist and could provide false positive results [2, 94].

Culture, still considered the "gold-standard", offers high sensitivity, up to 100% specificity and enables antimicrobial resistance (AMR) testing. Nevertheless, this method is relatively slow (relies on the growth kinetics of the organism), and requires strict conditions of specimen's collection, transportation and storage [2, 94].

The first molecular tests that were developed for *N. gonorrhoeae* were non-amplified nucleic acid hybridization (NAH) assays that relied simply on the binding of specific complementary nucleic acid probes and subsequent signal amplification to detect binding. Thus, throughout the last two decades, the clinical laboratory industry developed NAATs to detect *N. gonorrhoeae* with higher sensitivity than any prior diagnostic method, for which endocervical and vaginal swabs for women, urethral swabs for men, and urine specimens for both genders can be used, as well as pharyngeal and rectal specimens [2, 96]. NAATs are less demanding regarding specimen collection, transport and storage, detecting also nonviable gonococci, where noninvasive, self-collected samples can be effectively used. NAATs are faster, and as described for *C. trachomatis*, automation and robotics have been adapted for enabling a high throughput and simultaneous detection of several STIs; however, NAATs cannot completely replace culture, as AMR testing is not possible [2, 13, 94]. Commensal *Neisseria* species exhibit a high level of genetic similarity to *N. gonorrhoeae* and some early NAATs failed to differentiate *N. gonorrhoeae*, resulting in false-positive reports; modern NAATs overcome that limitation and specificity is now close to 100% [94].

Gonorrhea has been successfully treated by using antimicrobial drugs for the last 70 to 80 years. However, treatment of *N. gonorrhoeae* infections has successively failed because of its rapid ability to develop resistance mechanisms, leading to a high prevalence of *N. gonorrhoeae* strains with resistance to most of the antimicrobials available for treatment. This situation raises a concern about the potential emergence of untreatable gonococcal infections, with serious sexual and reproductive health consequences [97]. Ceftriaxone remains the last option for empirical antimicrobial monotherapy, although a decreased susceptibility to the extended-spectrum cephalosporins is increasing, with several countries reporting treatment failures [94, 96]. Nowadays, a dual-antimicrobial therapy is recommended, ceftriaxone plus azithromycin, based on the experience from other bacteria, where the use of two antimicrobials with different mechanisms of action improved treatment efficacy, and potentially slowed the emergence and spread of resistance. Furthermore, since people with gonococcal infection are frequently co-infected with *C. trachomatis*, the dual therapy helps solving both infections [96, 97]. Efficient patient management is important to control the spread of antimicrobial resistance, namely by increasing the awareness about the correct use of antibiotics (among healthcare providers and consumers), sending messages and making information available, by implementing interventions and appropriate treatment regimens, and by developing molecular methodologies for monitoring and detecting AMR [108, 109].

Development of novel prevention options for gonorrhea could be of interest. Petousis-Harris, H. et al., 2017 [110] consider that a vaccine with 30% efficacy could decrease the prevalence of gonorrhea by more than 30% within 15 years, if immunity is maintained. This would provide substantial public health benefits, and would surely contribute to achieve 90% reduction of gonorrhea incidence by 2030, relative to the incidence in 2018 [5].

4. *Mycoplasma genitalium*

Mycoplasma genitalium and *Mycoplasma hominis* belong to the genus *Mycoplasma* and, together with *Ureaplasma urealyticum* and *Ureaplasma parvum* (both previously known as *U. urealyticum*) they belong to the class Mollicutes [85].

4.1. Biological and genomic features

M. genitalium was firstly described in 1981 after isolation from urethral specimens from men with non-gonococcal urethritis (NGU) [111]. They are the smallest known free-living species of bacteria, with 580 073 base pairs [112] and a size usually ranging from 0.3 to 0.5 µm [113]. This organism lacks a cell wall making it inherently resistant to beta-lactam antibiotics, living in the ciliated epithelial cells of the urinary and genital tracts of humans [32, 113].

During evolutionary processes, *M. genitalium* lost considerable portions of their ancestors' chromosomes but retained the genes essential for life, such as the genes necessary for DNA replication, transcription and translation. As such, they had to develop multiple genetic systems in order to be able to acquire the necessary components from the host – making them host-dependents – such as nutrients like glucose and fructose, attachment organelles and adhesins [112, 114].

4.2. Epidemiology and pathology

In population-based studies, *M. genitalium* is found in 1% – 3% of sexually active men and women, according to the WHO [2], possibly explained by a greater number of "exposure" episodes [115, 116].

As for other STIs, *M. genitalium* infection occurs mostly in women of younger age, with associated risk factors such as having multiple sex partners during the past 6 months, bacterial vaginosis, a shorter duration of steady relationship and having a partner with symptoms [115, 117, 118, 119, 120]. *M. genitalium* infection in women is often asymptomatic, but it is related with an increased risk of cervicitis, pelvic inflammatory disease, infertility, and preterm delivery infected [4, 32, 85].

In men, *M. genitalium* infection has been associated with acute and chronic NGU (approximately in 25% of the cases) [9, 85, 113]. *M. genitalium* has also been found in the rectum, although not frequently causing rectal symptoms nor proctitis; even though, *M. genitalium* infection was more often detected in MSM than in heterosexual men [4, 121].

M. genitalium should be suspected in case of persistent or recurrent urethritis and may be considered in persistent or recurrent cases of cervicitis and PID [4]. Being a slow-growing organism unable to grow in artificial media, *M. genitalium* may take up to 6 months to be isolated in cell culture; thus, culture is not a reliable option for clinical diagnosis, which should be based on NAATs [2, 4, 32, 85, 122].

Recommended treatment consists in azithromycin or doxycycline [4, 123]. The former was considered the most effective, but resistance to azithromycin seems to be rapidly emerging [2, 113]. In some settings, treatment failures could occur in approximately 50% of all *M. genitalium* infections, due to macrolide resistance developed upon a prior treatment with a single dose of azithromycin [2, 85, 124, 125]. Moxifloxacin can be successfully used to treat *M. genitalium* in the case of treatment failure, with cure rates of 100% [125, 126].

A study [127] showed that women diagnosed with *M. genitalium* were at threefold higher risk of acquiring HIV in the 3 months following *M. genitalium* detection, due to the disruption of the mucosal barrier [128]. On the other hand, HIV itself or behavioral risks of HIV-infected women may favor the acquisition of *M. genitalium* infection [129].

Coinfection with *C. trachomatis* and/or *N. gonorrhoeae* is not uncommon in *M. genitalium* infected individuals [120, 130].

5. *Trichomonas vaginalis*

The flagellated protozoan *T. vaginalis* only infects humans, being the etiological agent of trichomoniasis [131].

5.1. Biological and genomic features

T. vaginalis is an obligate parasite that phagocytoses bacteria, vaginal epithelial cells, spermatozoids and erythrocytes [131]. Belonging to the family Trichomonadidae, *T. vaginalis* is a motile, ovoid, pear-shaped organism that measures approximately 10 – 20 µm (long) provided with four free, anterior flagella that enable twitching motility, fundamental to the survival in the vagina. A fifth flagellum embedded in an undulating membrane that assists in motility and to move extracellular nutrients towards the protozoan cytosol [2]. When in contact with the host epithelial cells and to attach to them, *T. vaginalis* internalizes the flagella and assumes an amoeboid conformation [132]. Attachment to the host epithelia is a crucial process for pathogenesis, largely mediated by a range of iron-dependent surface adhesins [133]. Adhesion leads to lysis of the host cell, erosion of the epithelial monolayer; this process makes available the necessary nutrients for *T. vaginalis* survival and replication [134].

This organism reproduces every 8 – 12 h, being unable to survive outside de host (only up to 30 minutes) because *T. vaginalis* doesn't have a cystic form, turning non-sexual transmission (contaminated showers, sharing of bathing implements, moist washcloths or toilet seats) merely hypothetical [135].

T. vaginalis has a repetitive genome with a size of ~160 Mb [136], with a high number of repeats that result in a highly fragmented sequence [137].

5.2. Epidemiology and pathology

Worldwide, trichomoniasis is one of the most common STIs, with an annual estimated incidence of 142 million [5]. In the United States, 7.4 million new cases are reported each year [138]. Depending on studies, the estimated prevalence of *T. vaginalis* greatly vary, from 2.3% among adolescents, 16% in women of reproductive age, 12.9% to 14.4% in high-risk populations of the same age group, 20.2% and 2% in African females and males, respectively, 22% and 2.2% in American females and males, respectively, and 5.8% and 0.6% in European women and men, respectively [134, 139, 140].

T. vaginalis is usually transmitted through vaginal sexual contact [141], being present in at least 80% of infected women and over 73% of their male partners [142]. Iatrogenic transmission is possible upon artificial insemination of contaminated semen [143]. In some rare cases, *T. vaginalis* might also be detected outside the genitourinary tract, in the pharynx and lower respiratory tract; neonates may also acquire infection vertically upon delivery, when the neonate passes through an infected birth canal [135].

The prevalence of *T. vaginalis* among females is higher than in men, being the female-to-male ratio of 4:1 to 10 times higher in women than in men, because of much more appropriate conditions of the vagina, in comparison to the urethra, for the protozoan growth [134, 144]. Thus, in men it usually presents as a more transient infection, which is generally not screened nor diagnosed [2, 145]. Contrasting with other STIs, *T. vaginalis* prevalence rates peak later in life, at 40 to 50 years old [146]. Many factors are known to influence the success of *T. vaginalis* infection, such as genetic variability of the isolates and the host immune response, as well as coexisting flora and sex hormones that regulate the menstrual cycle and thus the availability of iron in the genital tract [131]. Iron facilitates persistent infection among women, in contrast to men, in whom the absence of estrogen and the iron-depleted environment of the urethra surely render men less susceptible to infection [145].

T. vaginalis is not a reportable infection; however, screening should be considered for all individuals with high-risk behaviors, and for women who get tested for *C. trachomatis* or *N. gonorrhoeae* as an identification strategy [9, 145]. Partner notification should be done because of the high rates of concordant infections in couples.

T. vaginalis infections are asymptomatic in approximately 70 – 80% of men, and only 30 - 50% of women will develop symptoms in the 6-month period post-infection [144, 147, 148]. When present, symptoms overlap significantly the caused by a number of other sexually transmitted organisms. However, in women, infection can cause extensive changes in the vaginal microbiome causing bacterial vaginosis [149]. Other symptoms are vaginal discharge, that may be malodorous, airy and/or yellow-green, vagina and cervix with punctate hemorrhagic spots (''strawberry cervix''), vulvar irritation; *T. vaginalis* may also cause cervicitis, induces a 4.7-fold increase in the risk for developing PID, and be associated to cervical cancer and infertility [145, 150, 151, 152, 153]. Among pregnant women, *T. vaginalis* has been associated with preterm rupture of membranes, preterm delivery, and low-birth-weight neonates [154, 155]. In neonates, genital and nasopharyngeal infections have been reported [156].

Men might experience symptoms of urethritis, epididymitis, prostatitis and infertility through inflammatory damage or interference with sperm cell quality and function [4]. Zinc-rich environment of the prostate inhibits persistent infection, and so *T. vaginalis* is usually cleared spontaneously within approximately 10 days [145].

If left untreated, *T. vaginalis* can survive indefinitely in the lower urogenital tract, constituting a 2.7-fold increase in the risk of HIV acquisition and transmission, due to the local inflammatory response it evokes [2]. The incidence and severity of cervical dysplasia in women and of prostate cancer in men have also been associated with *T. vaginalis* [157].

Wet preparation microscopy of vaginal swab samples has been used for *T. vaginalis* diagnosis due to convenience, low cost and its applicability to be used in clinical settings together with testing for bacterial vaginosis. The sensitivity of the technique is low for vaginal exudates (60 – 70 %, and decreases by as much as 35 % within half hour after collection) and is even lower for urethral specimens of men [4, 158]. The low sensitivity relates with low organism load (below the limit of detection, as during asymptomatic infections), with temperature below 37ºC (implies the loss of motility soon after collection) and misidentification (caused by the often-present white blood cells of similar size). Thus, a well-trained technician is required for offering a diagnosis with an acceptable degree of specificity [2].

Culture is the most common and an appropriate method of diagnosis. Culture sensitivity is higher than that of microscopy, ranging from 44 to 95% while specificity is of up to 100 % [158, 159]. Nonetheless, the success of the method requires that biological samples are inoculated into culture medium immediately after collection [160].

At present, NAATs present the highest levels of sensitivity and specificity, being effective in detecting organisms from asymptomatic infections or at early phase of infection [9, 134]. NAATs are not limited by strict transport and temperature rules; other advantages include flexibility in types of samples, high throughput, and opportunity for automation, multiplexing, and quantification [2, 160].

Treatment is performed using nitroimidazoles and infected people and their parnterns are advised to abstain from sex until they are treated [135]. Although it has been stated [161] that treatment efficacy is influenced by vaginal ecology, a single dose of 2g of metronidazole or a 2g dose of tinidazole will cure more than 90% of infected women [4]. Both options are also recommended for men and the above-mentioned regimen of metronidazole is suitable for pregnant women. Despite the raising resistance to metronidazole, 1.7 to 10.1%, antimicrobial testing and test of cure are not recommended [162].

Objectives

The main objectives of the present dissertation are:

- To determine the prevalence of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis* in patients of the major Portuguese STD clinic (Unidade de consulta de DST/CAD da Lapa);
- To evaluate the distribution of *C. trachomatis ompA*-genotypes among the collection of the Portuguese National Institute of Health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, I.P.);
- To evaluate intra-patient *C. trachomatis* genetic heterogeneity affecting homopolymeric tracts potentially driving phase variation, among *C. trachomatis*-positive DNA samples selected from the collection of the Portuguese National Institute of Health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, I.P.).

Materials and Methods

1. Determining the prevalence of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis* in patients of the major Portuguese STD clinic

1.1. Biological samples and patient population

Between September 2016 and September 2017, 1034 endocervical, urethral, anorectal, oropharyngeal swabs and/or first-void of urine were collected from all patients attending an open STD primary clinic in Lisbon, Portugal (Unidade de consulta de DST/CAD da Lapa) and sent to INSA for routine diagnosis by Cobas® 4800 CT/NG (Roche Sistemas de Diagnóstico), belonging to a total of 987 patients.

Information such as age, gender, sexual orientation, number of partners, reason for attending the clinic, concurrent and previous STIs and HIV were also provided (**Table 2.1**) and inserted in an anonymous database of the host laboratory at INSA, IP. Oldest patient is 85 years old, with a mean age of 33 years old and mode of 26 years old.

Table 2.1 – Distribution of male and female samples per age, anatomical site, sexual orientation, number of sexual partners, reason for attendance, previous STIs and HIV. (*each person may have contributed with biological samples from different anatomical sites)

		Male samples*					**Female samples***				
		15–19	**20–24**	**25–34**	**35–44**	**45+**	**15–19**	**20–24**	**25–34**	**35–44**	**45+**
Anatomical site	Anorectum	2	8	10	7	1	0	0	0	0	0
	Endocervix/urethra	26	99	259	186	117	17	71	129	52	25
	Oropharynx	1	0	12	7	4	0	0	0	0	0
Sexual orientation	Homosexual	12	54	126	102	50	0	0	2	1	0
	Heterosexual	15	52	139	95	61	15	70	123	46	25
	Bisexual	2	1	16	4	11	2	1	4	5	0
Number of sexual partners	0	1	2	8	6	5	2	3	3	1	3
	1	11	29	66	50	38	8	31	64	31	18
	2 – 4	12	58	146	106	55	6	34	52	17	3
	>5	5	17	61	39	24	1	3	10	3	1
	Unknown	0	1	0	0	0	0	0	0	0	0
Reason for attendance	Screening	11	36	131	118	63	7	48	77	31	12
	Symptoms	18	71	150	83	59	10	23	51	21	13
Previous STIs	No	28	74	179	125	79	13	67	102	49	20
	Yes	1	33	102	76	43	4	4	27	3	5
HIV	Negative	27	103	259	174	105	17	70	129	51	24
	Positive	2	4	22	27	17	0	1	0	1	1

1.2. Real-time PCR

The eluates from Cobas® 4800 CT/NG testing were collected and conserved at -20ºC until testing, to avoid degradation of nucleic acids with PCR sensitivity decrease implications. Eluates (5µL per test) were tested by the S-DiaMGTV™ kit (Diagenode), a test that contains specific primers probes for the simultaneous amplification of *M. genitalium* and *T. vaginalis* genome in a single reaction (FAM (Ex./Em.: 494/520 nm) for the former and YELLOW (Ex./Em.: 530/549 nm) for the later). The test also includes positive and negative controls, and was performed according to the manufacturer's instructions (amplification mixture described in **Table 2.2** and real time PCR conditions described in **Table 2.3**) in the Lightcycler z480 from Roche, using the user-defined workflow (UDF) software.

Table 2.2 – PCR mix for S-Dia MGTV™ kit (final volume 25µL).

Reagent	Volume per sample
5x Concentrated MM	5 µL
MGTV double-dye probe and primers	2.5 µL
Water	12.5 µL

Table 2.3 – kPCR amplification profile for S-DiaMGTV™ kit.

Temperature	Time	Cycles
50ºC	2 minutes	1x
95ºC	10 minutes	
95ºC	15 seconds	45x
60ºC	1 minute	

Results were provided by the equipment by selecting the option "Absolute Quantification/Fit Points". The run was valid according to the described in **Table 2.4**.

Table 2.4 – Run validity testing.

Negative control	**No** FAM and Yellow signal above the threshold	Run is valid
	FAM and/or Yellow signal above the threshold	Run is invalid
Positive control	**No** FAM and Yellow signal above the threshold	Run is invalid
	FAM and/or Yellow signal above the threshold	Run is valid

1.3. Statistical analysis

Statistical analysis was performed with the software IBM® SPSS® Statistics 24.0.0.

Statistical analysis of the distribution of STIs results across the clinical data (age, gender, anatomical site, sexual orientation, number of sexual partners, reason for attendance, previous STIs and HIV) was performed by the Qui-square test.

Complementary to the referred test, measure of association Cramer's V (φ_c) was also performed.

Statistical significance, for all assays, was assumed for $p \leq 0.05$. When the *p-value* had very small values of magnitude, we assumed it as $p<<0.001$.

2. Evaluation of the distribution of *ompA*-genotypes among the collection of the Portuguese National Institute of Health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, I.P.)

2.1. Biological samples and patient population

C. trachomatis ompA-genotyping is routinely and systematically performed over all *C. trachomatis* positive samples (urines and endocervical, urethral, anorectal, conjunctival and oropharyngeal exudates kept in a sucrose-phosphate buffer, 2SP) detected in the INSA routine diagnosis or in routine diagnosis performed elsewhere and sent to INSA for national collection purposes.

Positive samples detected from November 2016 to June 2017 (n=374) were *ompA*-genotyped according to the methodology described in 2.2. For analysis purposes, these results were added to the obtained by others, in order to comprise the whole INSA's collection of *ompA*-genotyped *C. trachomatis* specimens, from 1990 to 2017.

2.2. DNA extraction and *ompA*-genotyping

DNA was extracted from *C. trachomatis* positive samples by using the NucliSENS®EasyMag (BioMérieux), according to manufacturer's instructions. For exudates, 200µL of a 2mL suspension in 2SP were used, while for urines 3mL were centrifuged for obtaining a pellet that is suspended in 200µL of PBS buffer. After extraction, DNAs were kept at -20ºC until use.

A nested-PCR technique was used to amplify the *C. trachomatis ompA* gene adapted from Lan, J. et al., 1993 [163]. This two sequential PCR amplification approaches aim to increase sensitivity. The primers used were NLO and NRO and PCTM3 and Sero2A (see **Annex 1)**, for the first amplification and the second amplification, respectively, and will promote an amplicon of ~1000 bp (see **Table 2.5** and **2.6**). Ten µL of DNA sample were used in the first PCR while 2µL of the first amplification product were used for the second PCR.

Table 2.5 – *ompA* nested-PCR reaction mixture for a final volume of 25µL.

Reagent	Volume per sample
dNTPs [a]	5 µL
Buffer (10x) [b]	2.5 µL
Magnesium Chloride ($MgCl_2$) (50mM) [b]	1.4 µL
Bio-x-act short polymerase (4U/mL) [b]	0.37 µL
Primers pair (25pmol/µL) [c]	2 µL
Water (Dnase, Rnase, Protease-free)	3.73 µL / 11.73 µL

[a] Applied Biosystems; [b] Bioline; [c] Invitrogen.

Table 2.6 – *C. trachomatis ompA* nested-PCR amplification profile.

1st PCR (Temperature)	2nd PCR (Temperature)	Time	Cycles
95ºC	95ºC	5 minutes	1x
55ºC	50ºC	1 minute	
72ºC	72ºC	1'25 seconds	35x
95ºC	95ºC	30 seconds	
55ºC	50ºC	30 seconds	
72ºC	72ºC	10 minutes	1x

Amplicon detection was performed after the second round of amplification, by 1% (w/v) agarose gel electrophoresis, using TAE buffer and SYBR® Safe DNA gel stain (10 000x in DMSO; Invitrogen).

Whenever a positive *ompA* nested-PCR product was observed, it was purified using ExoSAP® (Affymetrix) in a two-step reaction, 37ºC for 15 minutes plus 80ºC for 15 minutes. The kit contains two enzymes (Exonuclease I combined with Shrimp Alkaline Phosphatase) capable of removing contaminants; thus, the first cycle aims to activate the enzymes that will be the inactivated during the second step.

Finally, purified amplicons were sequenced; sequencing reaction was prepared as described in **Table 2.7**, using the set of primers described in **Annex 2** for a final volume of 9,5 µL, to which was added 0,5 µL of purified PCR product. Big-Dye technology was used, consisting in four dideoxynucleotide terminators tagged with a different fluorescent dye each **[164]**.

Table 2.7 – Sequencing mix.

Reagent	Volume per sample
Big-Dye [a]	2.5 µL
Sequencing buffer (5x) [a]	0.75 µL
Each primer (5 pmol/µL) [b]	1 µL
Water (Dnase, Rnase, Protease-free)	5.25 µL

[a] Applied Biosystems; **[b]** Invitrogen

The amplification profile for the sequencing reaction is described in **Table 2.8**.

Table 2.8 – Sequencing profile.

Temperature	Time	Cycles
96ºC	30 seconds	1x
96ºC	10 seconds	25x
50ºC	5 seconds	
60ºC	1 minute	

Sanger sequencing was performed by the Unit of Technology and Information (UTI) of INSA. Sequences were analyzed using Chromas Lite software (Technelysium Pty Ldt), and were further aligned with *ompA* sequences of *C. trachomatis* prototype strains, representing all *ompA*-genotypes, and with variants previously identified in the laboratory. Alignments, phylogenetic analyses and pairwise comparisons were done using the DNASTAR's Lasergene Molecular Biology Suite available at INSA.

Whenever a new variant was detected, the whole *ompA*-genotyping procedure was repeated in order to confirm the observed mutation(s).

2.3. Statistical analysis

Statistical analysis was performed with the software IBM® SPSS® Statistics 24.0.0.

Statistical analysis of the distribution of *C. trachomatis ompA*-genotypes across the clinical data (anatomical site and age) was performed by the Qui-square test.

Complementary to the referred test, measure of association Cramer's V (φ_c) was also performed.

Statistical significance, for all assays, was assumed for $p \leq 0.05$. When the *p-value* had very small values of magnitude, we assumed it as $p<<0.001$.

3. Evaluation of intra-patient *C. trachomatis* genetic heterogeneity affecting homopolymeric tracts potentially driving phase variation, among *C. trachomatis*-positive DNA samples selected from the collection of the Portuguese National Institute of Health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, I.P.)

3.1. Biological samples and patient population

C. trachomatis-positive DNA samples (n=96) were selected from the laboratory database, including samples from anorectal, endocervical/urethral, ocular and oropharyngeal exudates. All DNA samples were directly obtained from clinical specimens, so no culture was performed. Selection was performed in order to cover multiple types of exudates and serovars.

To reinforce the dataset, we further included a set of 71 samples for which whole-genome sequencing data has been recently released by Hadfield, J. et al, 2017 [48]. In fact, we could take advantage of this study as the sample-type characteristics and methodology fit the above key criterium, i.e., the associated DNA samples were extracted directly from clinical specimens. In summary, a total of 167 *C. trachomatis*-positive samples (**Table 2.9**) were evaluated regarding the intra-patient variability of homopolymeric tracts.

Table 2.9 – Distribution of biological samples per anatomical site and *ompA*-genotype.

		***ompA*-genotype**												
		B	**D**	**Da**	**E**	**F**	**G**	**H**	**Ia**	**J**	**Ja**	**K**	**L1**	**L2**
Anatomical Site	Anorectum	0	0	5	2	1	5	0	0	4	0	0	0	11
	Conjunctiva	0	0	3	3	2	0	0	0	0	0	0	0	0
	Endocervix/Urethra	1	11	2	32	22	5	4	7	7	3	9	1	0
	Oropharynx	0	0	1	2	0	4	0	0	2	0	0	0	0
	Unknown	0	3	0	10	4	1	0	0	0	0	0	0	0

3.2. Homopolymeric tracts evaluation

In a preliminary assay, the Bioinformatics Unit of the Department of Infectious Diseases of INSA had previously developed, optimized and validated a strategy focused on sequencing, through a high throughput next-generation sequencing (NGS) technology, amplicons obtained with PCR schemes targeting specific *C. trachomatis* genome-dispersed homopolymeric tracts (poly (Ns)). After this preliminary survey, it has been possible to identify 20 homopolymeric tracts likely prone to yield intra-patient allelic variation with potential impact on phenotype (the poly (N) location in relation to the potentially affected genes (upstream or coding region) and the location of the gene were taken into account).

In the present study, we took advantage of the recent availability of NGS data collected directly from clinical samples (Hadfield, J. et al., 2017 [48]) to refine the selection, ending up with a total of 12 potentially heterogeneous poly (N) to be evaluated. These were scrutinized at bioinformatics level after amplicon-based NGS (96 samples from INSA) or directly from the publicly available data (71 samples from Hadfield, J. et al., 2017 [48]) (see next section). **Annex 3** details the poly (N) location in relation to the potentially affected genes (upstream or coding region), while **Annex 4** provides the complete list of samples under study.

3.3. Amplicon-based NGS and bioinformatics analysis

PCR primers targeting each one of the poly (Ns) selected were previously designed (**Annex 5**) for the preliminary study and oligonucleotides were ordered, complemented with Illumina adapter sequences at their 5' ends (**Annex 6**).

To amplify each homopolymeric region, a PCR technique with DNA polymerase KAPA HiFi HotStart ReadyMix PCR Kit from Roche – an enzyme recognized by minimizing polymerase induced errors, was employed according manufacturer's instructions. Each PCR generates an amplicon of ~250 bp.

PCR mixtures were prepared for a total volume of 22.5 µL (**Table 2.10**) to which 2.5 µL of each DNA is added afterwards.

Table 2.10 – PCR mix for homopolymeric targets.

Reagent	Volume per sample
KAPA HiFi Polymerase [a]	12.5 µL
Primers pair (5pmol/µL) [b]	2 µL
Water (Dnase, Rnase, Protease-free)	8 µL

[a] Roche; [b] Invitrogen

The amplification profile comprehends three steps, starting with the denaturation. This step is followed by 35 cycles of denaturing, annealing and extension, ending with a final extension step, according to **Table 2.11**. Annealing temperature varies for each PCR schema (**Annex 7**) used and were previously optimized in the preliminary study.

Table 2.11 – Amplification profile for homopolymeric regions.

Temperature	Time	Cycles
95ºC	3 minutes	1x
95ºC	30 seconds	
Annealing temperature*	30 seconds	35x
72ºC	30 seconds	
72ºC	5 minutes	1x

* Temperature adjusted for each target

For each clinical isolate, amplicons generated (for all gene targets) are pooled into a single tube and sent to the UTI – INSA, where they are cleaned-up, indexed and subjected to cluster generation and paired-end sequencing (2x150 bp) in an Illumina MiSeq equipment (Illumina Inc., San Diego, CA, USA), according to manufacturer's instructions.

The Bioinformatics Unit used an in-house Phyton script (described in Pinto, M. et al., 2016 [165]) in order to evaluate the in-length variation of DNA homopolymeric tracts. Briefly, the script enables the extraction and counting (directly from raw reads, both forward and reverse) of DNA sequences that are contiguously flanked by two conserved, user-defined, small DNA strings (**Figure 2.1**), being able to determine the precise relative frequency of clones carrying specific base counts within each given sample. As previously performed [165], homopolymeric tracts were classified as 'variable' in a given sample if the dominant 'count' represented less than 90% of all respective reads (Forward + Reverse) counted in that region.

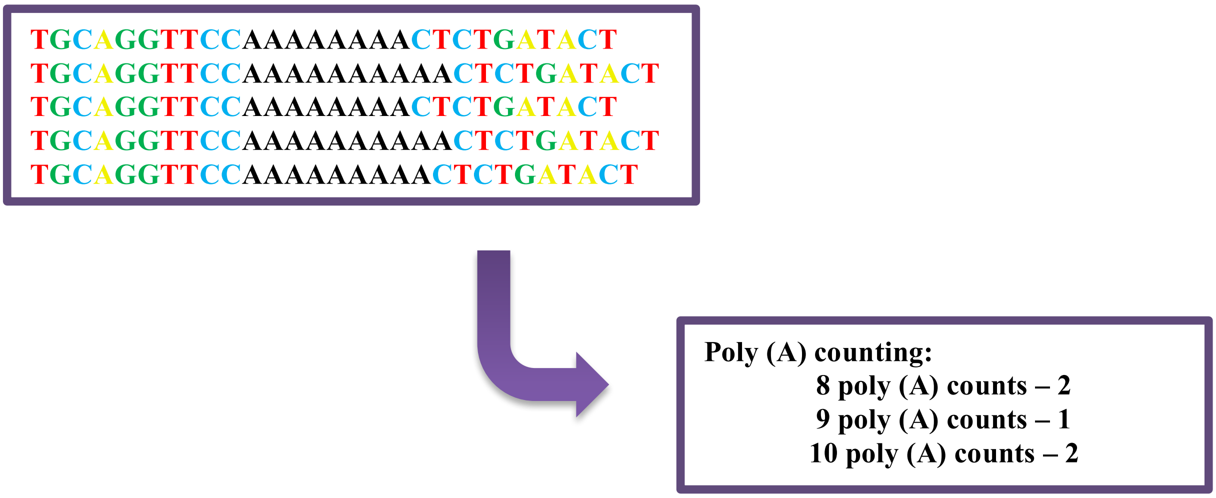


Figure 2.1 – Schematic representation of homopolymeric tracts counts.

Results

1. Determination of the prevalence of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis* in patients of the major Portuguese STD clinic (Unidade de consulta de DST/CAD da Lapa)

A total of 1034 samples were involved in this study, 740 (71.6%) were from men and 294 were from women (28.4%). Almost two thirds of the population reported having more than one sexual partner during the last six months. Endocervical/urethral samples constituted the great majority (n=982, 95.0%), followed by anorectal (n=28, 2.7%) and oropharyngeal samples (n=24, 2.3%).

For analysis purposes regarding sexual orientation, two groups were defined: heterosexuals and MSM (including both homosexual and bisexual men), since only 5.1% (15/294) of samples from women were from homosexual or bisexual.

Two hundred and eight STIs cases were detected (*C. trachomatis* = 108, 51.9%; *N. gonorrhoeae* = 68, 32.7%; *M. genitalium* = 27, 13.0%; *T. vaginalis* = 5, 2.4%) (**Figure 3.1**), in 186 (18.8%) individuals; thus, 19 (10.2%) individuals (10 men and 9 women) were coinfected with more than one of the STIs under evaluation.

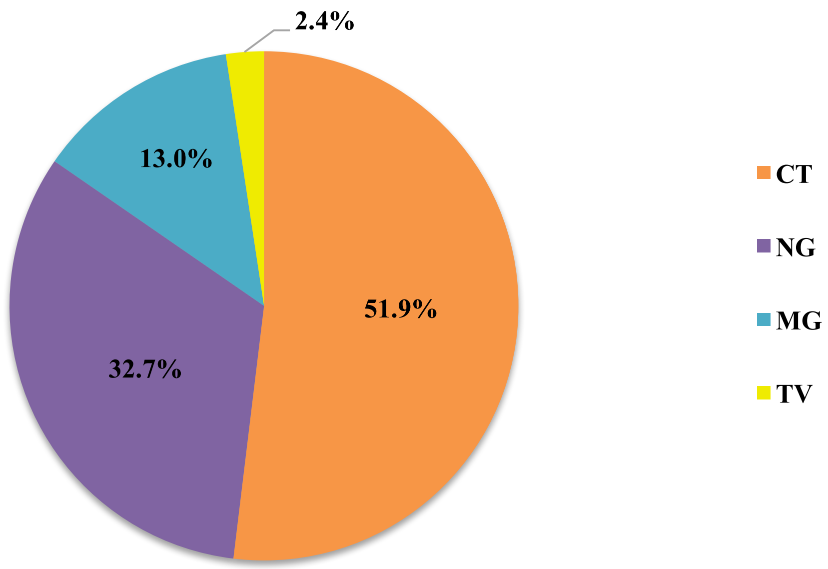


Figure 3.1 – Distribution (in %) of the diagnosed STIs (CT – *C. trachomatis*; NG – *N. gonorrhoeae*; MG – *M. genitalium*; TV – *T. vaginalis*).

C. trachomatis was the most prevalent STI in the studied population, occurring in 10.4% (108/1034), according to **Figure 3.2**; 10.1% (75/740) of the men were infected by *C. trachomatis* as well as 11.2% (33/294) of the women.

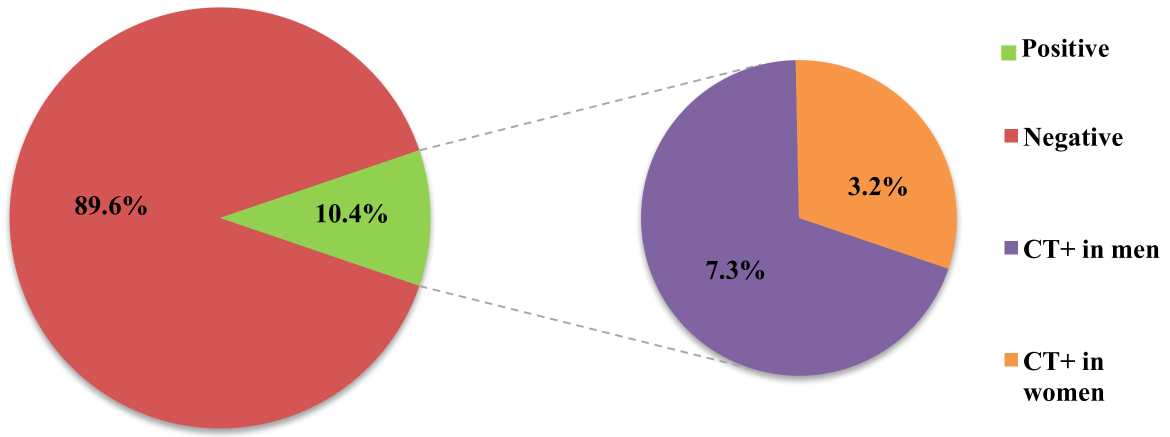


Figure 3.2 – Prevalence of *C. trachomatis* (CT) in the studied population and distribution (in %) of CT+ by gender.

Chlamydial infection was detected in all age classes (**Figure 3.3**), being there a statistically significant association ($p=0.015$, $\varphi_c=0.109$). This STI was the most frequent among 25 to 34 years old people (n=49, 45.4%), followed by the 20 to 24 years group (n=28, 25.9%), the 35 to 44 years group (n=19, 17.6%), 45 years and above (n=9, 8.3%) and finally the group 15 to 19 years (n=3, 2.8%).

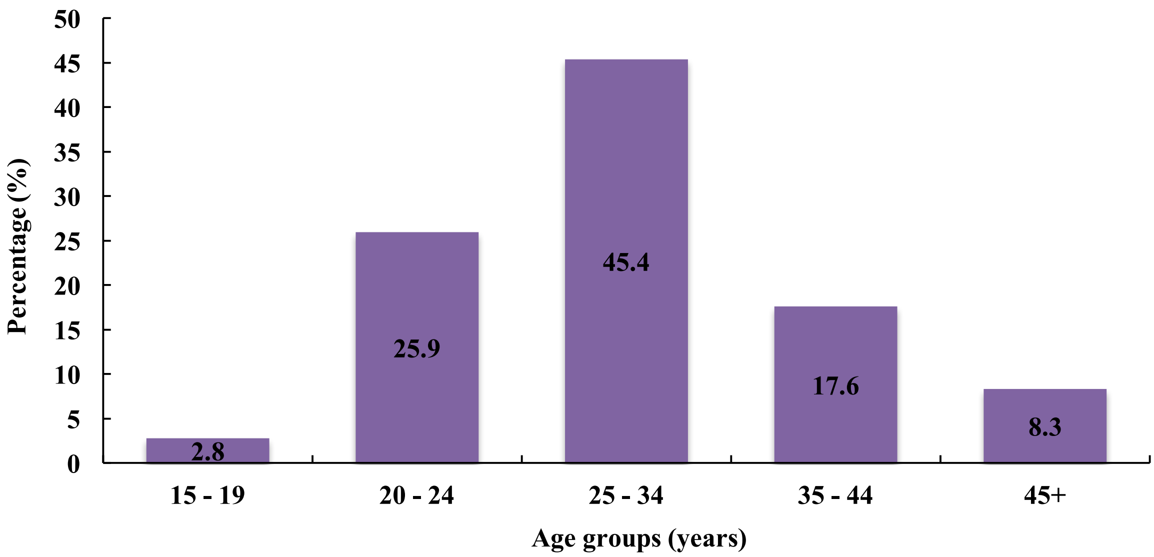


Figure 3.3 – Frequency of *C. trachomatis* infection according to age groups.

C. trachomatis could be detected in all anatomical sites. Endocervical/ Urethral samples contributed with 101 (93.5%) positive cases, six anorectal and one oropharyngeal case, which altogether represented 6.5% of the chlamydial infections. However, although the number of samples per anatomical site is not even, it is of note that *C. trachomatis* was present in 101/982 (10.3%) endocervical/urethral samples, 6/28 (21.4%) anorectal samples and in 1/24 (4.2%) oropharyngeal samples.

More than two thirds (n=74, 68.5%) of the *C. trachomatis* infected patients had more than one sexual partner during the last six months. Only one (0.9%) infected patient reported no recent sexual partner and the remaining 33 patients (30.6%) reported having one sexual partner **(Figure 3.4)**.

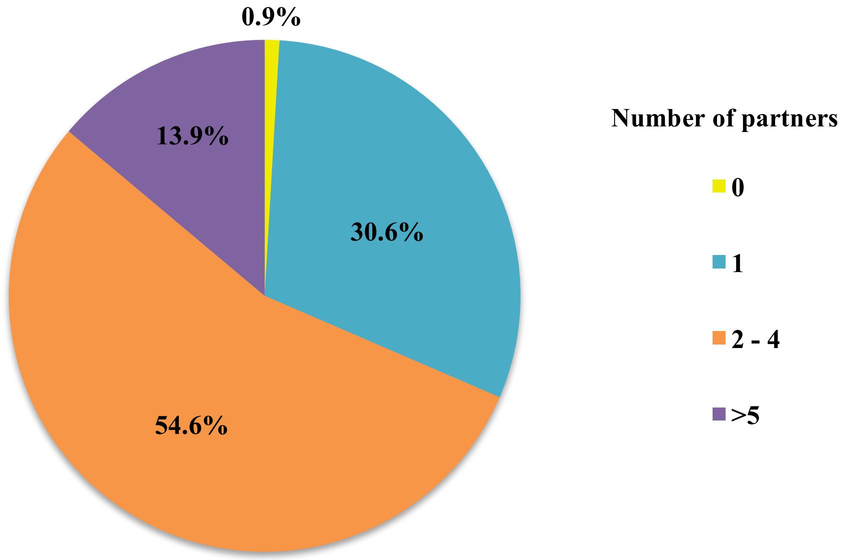


Figure 3.4. – Distribution (in %) of *C. trachomatis* infections according to patient's number of sexual partners.

Regarding sexual orientation, 76.9% (n=83) of the infected with *C. trachomatis* were heterosexual, while the remaining reported being MSM (n=25, 23.1%). However, as the distribution of individuals per sexual orientation is not even, it is of note that *C. trachomatis* was present in 83/641 (12.9%) heterosexual individuals and in 25/378 (6.6%) MSM, being there a statistically significant association between chlamydial infection and sexual orientation ($p=0.002$, $\varphi_c=0.077$).

Of all asymptomatic individuals, 52/534 (9.7%) were infected with *C. trachomatis*, while among symptomatic 56/500 (11.2%) were also infected by *C. trachomatis*; there was no statistically significant association between 'having symptoms' and being infected ($p=0.442$).

C. trachomatis was also found in co-infection with *N. gonorrhoeae*, which was the second most frequently detected STI, 6.6% (68/1034). *N. gonorrhoeae* was most commonly found in men (52/740, 7.0%) than in women (16/294, 5.4%).

As described for *C. trachomatis, N. gonorrhoeae* is present along all age groups, being more frequent among young adults (20 – 34 years old, 79.4%). The 25 to 34 years group was the most affected by *N. gonorrhoeae* (n=30, 44.1%), followed by the 20 to 24 years group (n=24, 35.3 %), the 35 to 44 years group (n=9, 13.2 %), 15 to 19 years (n=3, 4.4 %) and finally the 45 years and above group (n=2, 2.9 %) (**Figure 3.5**).

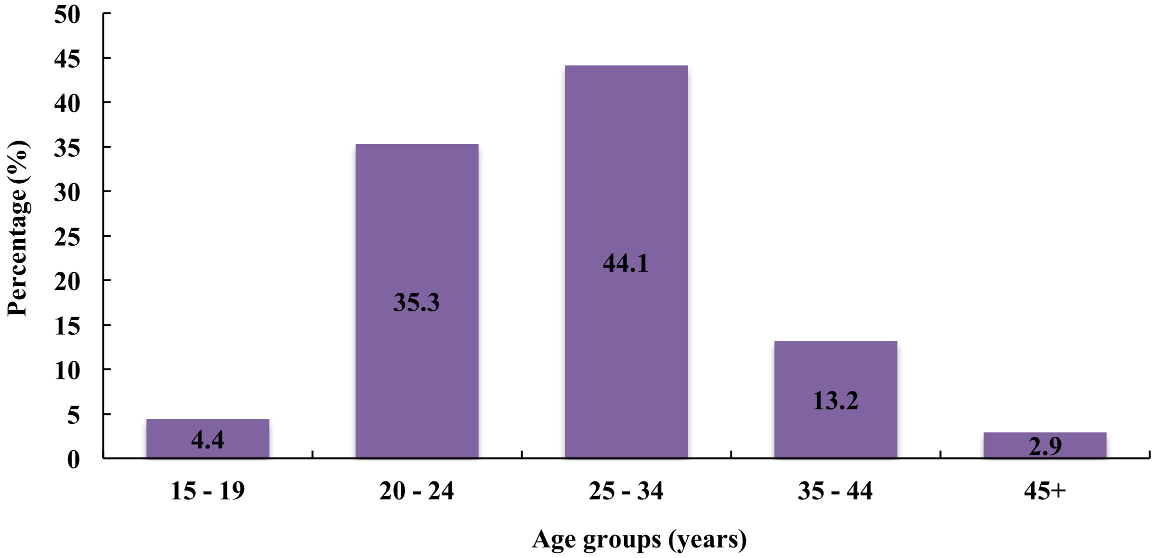


Figure 3.5 – Distribution of *N. gonorrhoeae* infected patients according to age groups.

As for *C. trachomatis*, *N. gonorrhoeae* was detected in all anatomical sites, namely the endocervix/urethra (58/68, 85.3%), the rectum (8/68, 11.8%) and the oropharynx (2/68, 3.0%). Although *N. gonorrhoeae* was present in 58/982 (5.9%) endocervical/urethral samples, in 8/28 (11.8%) anorectal samples and in 2/24 (8.3%) oropharyngeal samples, as explained above for *C. trachomatis*, the number of samples per anatomical site is not even. However, there is a statistically significant association with between the *N. gonorrhoeae* infection and the anatomical site ($p<<0.001$, $\boldsymbol{\varphi}_c=0.376$).

Again, almost three quarters of the *N. gonorrhoeae* infected patients (50/68, 73.6%) reported having more than one sexual partner for the last six months, whereas 25.0% (17/68) reported having only one sexual partner (**Figure 3.6**).

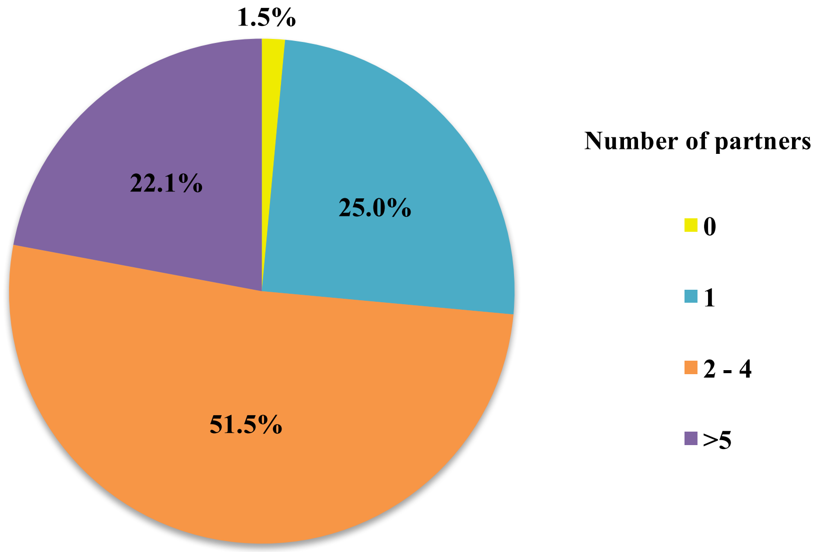


Figure 3.6 – Distribution (in %) of *N. gonorrhoeae* infections according to patient's number of sexual partners.

Contrarily to what was observed for *C. trachomatis* infected patients, here we observed that the frequency of gonococcal infections among heterosexual individuals was lower than half (31/68, 45.6%). The remaining 54.4% (n=37) were MSM. Although the distribution of individuals is not even, it is of note that *N. gonorrhoeae* was present in 31/641 (4.8%) heterosexual individuals and in 37/378 (9.8%) MSM and that there's a statistically significant association ($p=0.002$, $\varphi_c=0.096$).

Asymptomatic *N. gonorrhoeae* infected patients, 16/534 (3.0%), were less frequent than symptomatic 52/500 (10.4%), with a statistically significant association between character of infection and *N. gonorrhoeae* infection ($p<<0.001$, $\varphi_c=0.149$).

The frequency of *M. genitalium* was 2.6% (27/1034); 2.6% (19/740) of the men were infected as well as 2.7% (8/294) of the women.

As for *C. trachomatis* and *N. gonorrhoeae* infected patients, *M. genitalium* was most common among individuals aged 20 to 34 years old (18/27, 66.7%), as 12 (44.4%) cases occurred in people aged 25 to 34 years and 6 (22.2%) in the 20 to 24 years age group; nonetheless, 5 (18.5%) cases were detected in the 35 to 44 years group, 3 (11.1%) in the individuals aged 45 years and more and a single case (3.7%) in youngs aged 15 to 19 years (**Figure 3.7**).

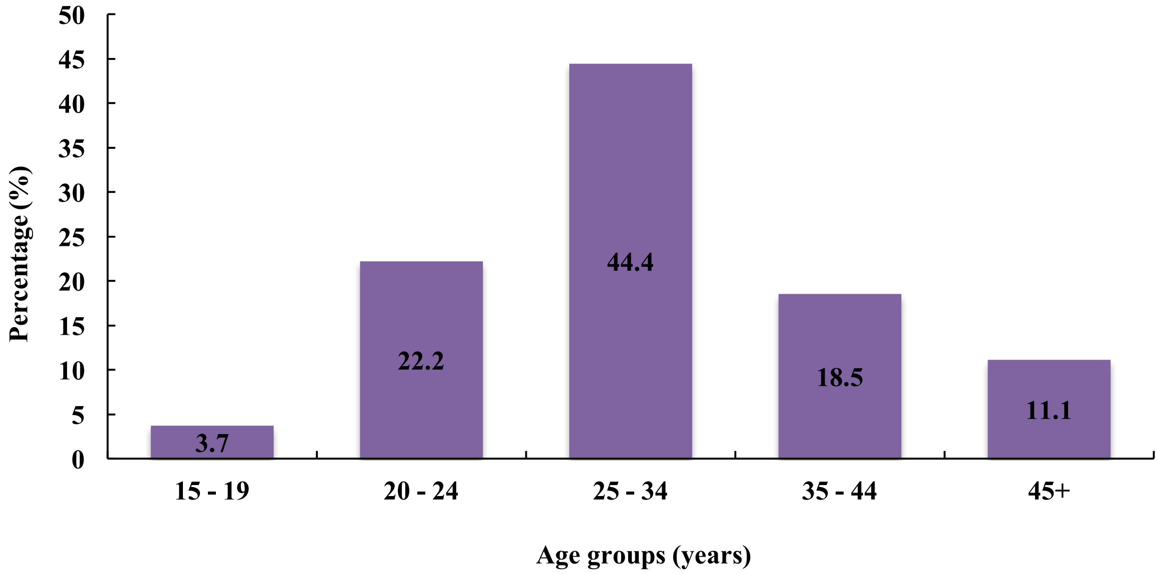


Figure 3.7 – Distribution of *M. genitalium* infected patients according to age groups.

Most of the *M. genitalium* cases (26/27, 96.3%) were detected in the endocervix/urethra and one in anorectal exudates and no cases detected among oropharyngeal samples. However, as previously stated, the anatomical site from where STI diagnosis were more often required was the endocervix/urethra, and for the ensemble of these samples *M. genitalium* was only present in 2.6% (26/982) of the endocervical/urethral samples, being more frequent in anorectal exudates (1/28, 3.6%).

Most of *M. genitalium* infected patients reported having more than one sexual partner during the last six months (19/27, 70.4%), while the remaining 8 (29.6%) patients reported having only one partner (**Figure 3.8**).

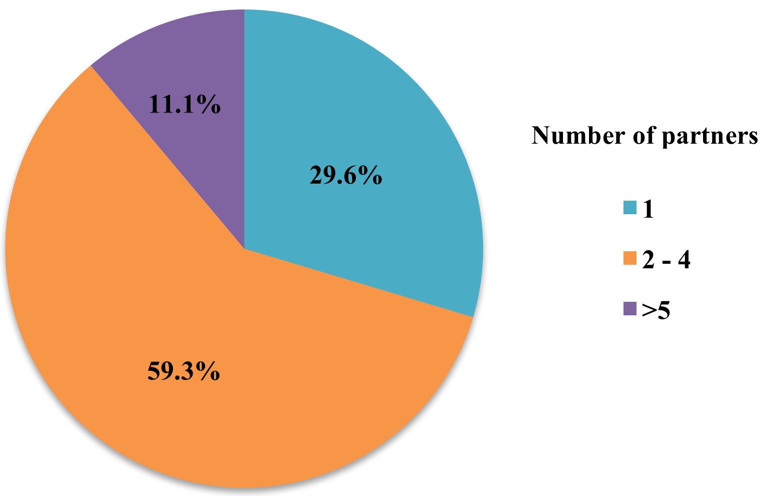


Figure 3.8 – Distribution (in %) of *M. genitalium* infections according to patient's number of sexual partners.

According to sexual orientation, 55.6% (15/27) of *M. genitalium* infected patients reported as being heterosexual or MSM (12/27, 44.4%). Again, the distribution of individuals by sexual orientation is not even; *M. genitalium* was present in 15/641 (2.3%) heterosexual individuals and in 12/378 (3.2%) MSM.

M. genitalium was as frequent among symptomatic patients as among asymptomatic, 3.2% (16/500) and 2.1% (11/534), respectively.

Of all STIs under evaluation, *T. vaginalis* was the less frequent 0.5% (5/1034), with most infected being women (4/294, 1.4%); in men, it only represented 0.1% (1/740).

T. vaginalis infected patients' age was higher, with 60.0% (3/5) having 35 to 44 years; in people aged 20 to 24 one case was detected, and the same number in the 25 to 34 years age group. In the remainder (15 to 19; above 45) no cases were detected.

The urethra was the only anatomical site where *T. vaginalis* infection was detected.

Eighty percent (4/5) of the *T. vaginalis* infected patients reported having two to four sexual partners for the last six months; the fifth case was detected in a patient declaring having had only one partner.

None of the *T. vaginalis* infected patients reported as being homosexual; four identified themselves as heterosexual and one as bisexual.

Also, none of the *T. vaginalis* infected patients went to consultation due to symptoms, but only due to screening purposes. In general, among all the patients that went for this purpose, 5/534 (0.9%) were infected with this microorganism.

2. Evaluation of the distribution of *C. trachomatis ompA*-genotypes among the collection of the Portuguese national institute of health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, IP)

2.1. *ompA*-genotype distribution at INSA's collection

Twelve *ompA*-genotypes were ever identified among the INSA's collection of 2579 *C. trachomatis* positive samples. The most represented *ompA*-genotype was E (n=862, 33.4%), followed by F (n=428, 16.6%), D (n=402, 15.6%) and G (n=286, 11.1%). Less represented were *ompA*-genotypes J (n=165, 6.4%), I (n=161, 6.2%), L2 (n=99, 3.8%), H (n=76, 2.9%), K (n= 49, 1.9%), B (n=11, 0.4%) and C (n=3, 0.1%); mixed infections could be detected in 1.4% (n=36) of the cases and, for the last 27 years, *ompA*-genotype L1 was detected once. *ompA*-genotypes A and L3 have never been identified (**Figure 3.9**).

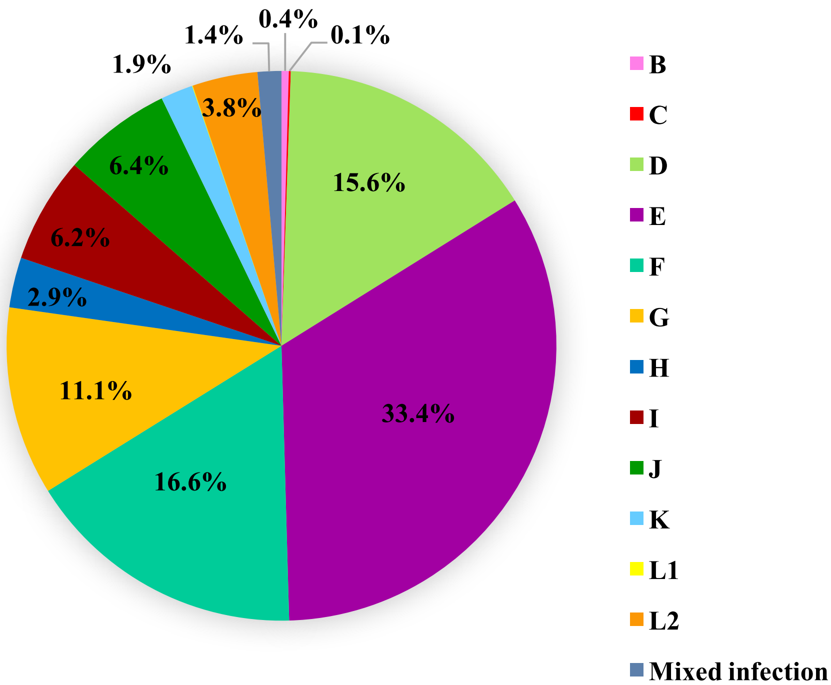


Figure 3.9 – Distribution (in %) of *ompA*-genotypes in INSA's *C. trachomatis* positive sample collection.

There is a statistically significant association between *ompA*-genotypes and the anatomical site of infection ($p<<0.001$, $\varphi_c=0.379$). *ompA*-genotypes D and E were detected in all anatomical sites of infection and F, G and J were also present in most of them (**Figure 3.10** and **Annex 8**). Genotype L2 was the most common in the anorectum. The endocervix/urethra was the only anatomical site where all *ompA*-genotypes were present.

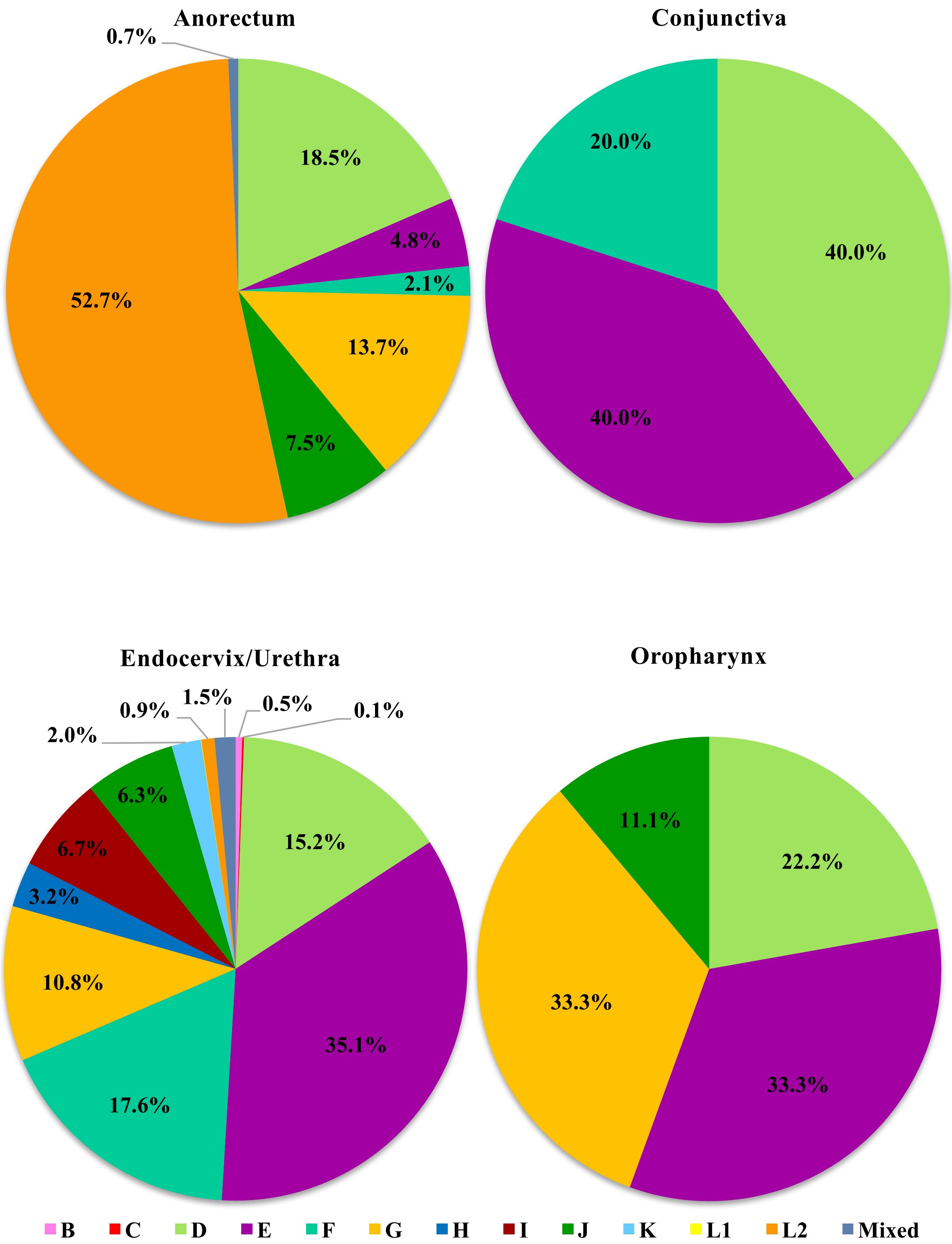


Figure 3.10 – Distribution (in %) of *ompA*-genotypes per anatomical site of infection.

The most common *ompA*-genotype in both genders was the E. F was the second most common among men, while for women, it was D. *ompA*-genotypes G and L2 are much more common among men than among women (**Figure 3.11** and **Annex 9**).

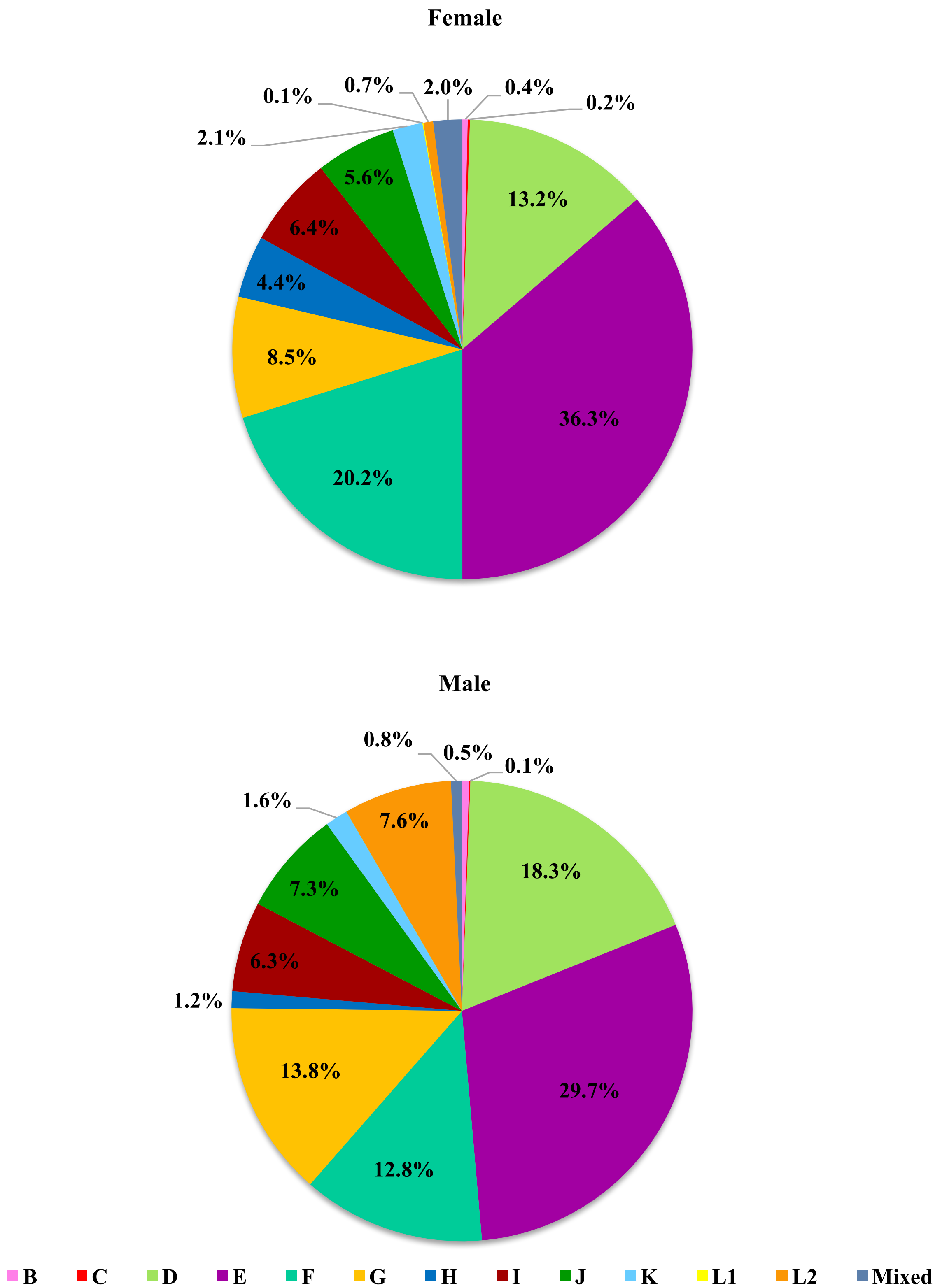
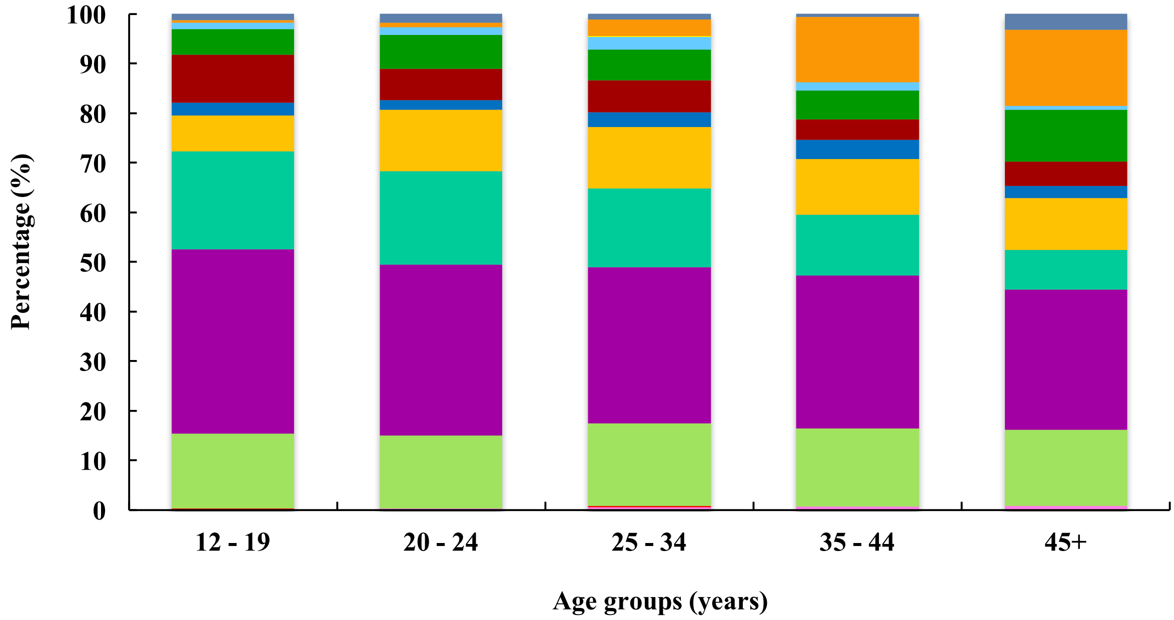


Figure 3.11 – Distribution (in %) of *ompA*-genotypes per gender.

The frequency of *ompA*-genotypes among age groups is presented in **Figure 3.12 a)** and **Annex 10**. L2 was more common among patients with 25 years and older (**Figure 3.12 b**).

a)



b)

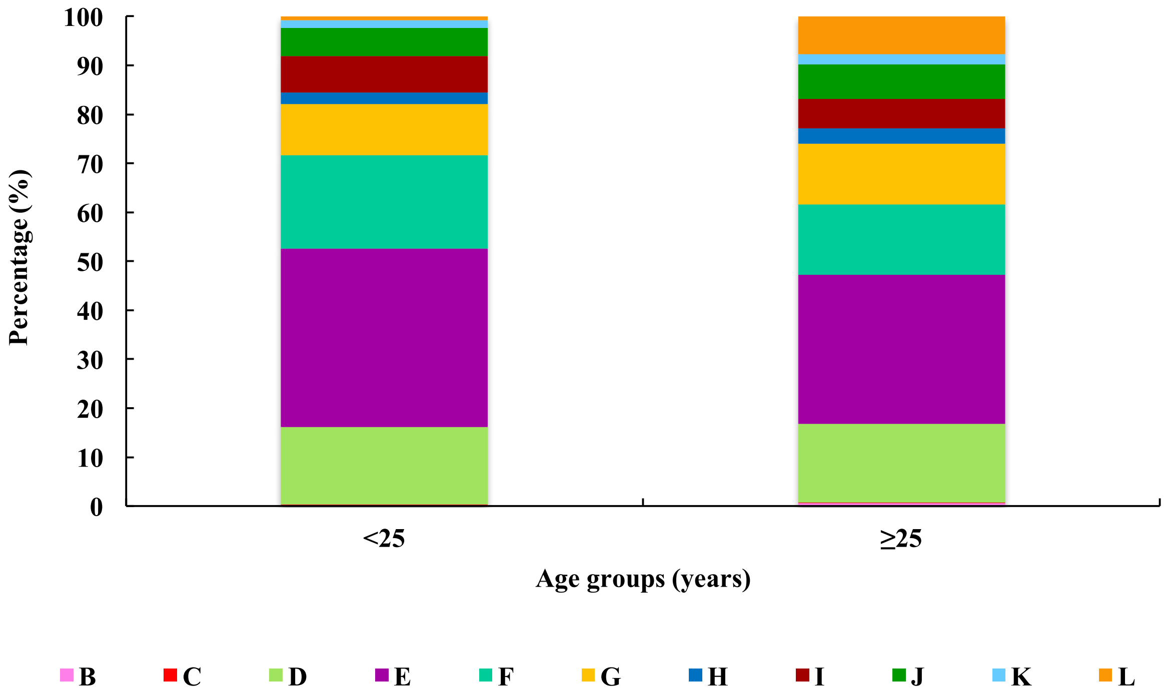


Figure 3.12 – Distribution of *ompA*-genotypes per age.

ompA-genotype frequency along the years is shown in **Figure 3.13** and **Annex 11**.

ompA-genotype B appeared in the year 1994 and is regularly detected, although always by a very limited number of strains. *ompA*-genotype C was only detected in the year 2003.

ompA-genotype D was the first to be detected at INSA, back in the year 1990, and has been identified ever since, being one of the most common genotypes, as genotype G; however, not as frequent as F and specially, E.

Regarding LGV *ompA*-genotypes, L3 was never detected, L1 appeared only once, in the year of 1994, whereas L2 has become quite common since 2007, in contrast to prior decades.

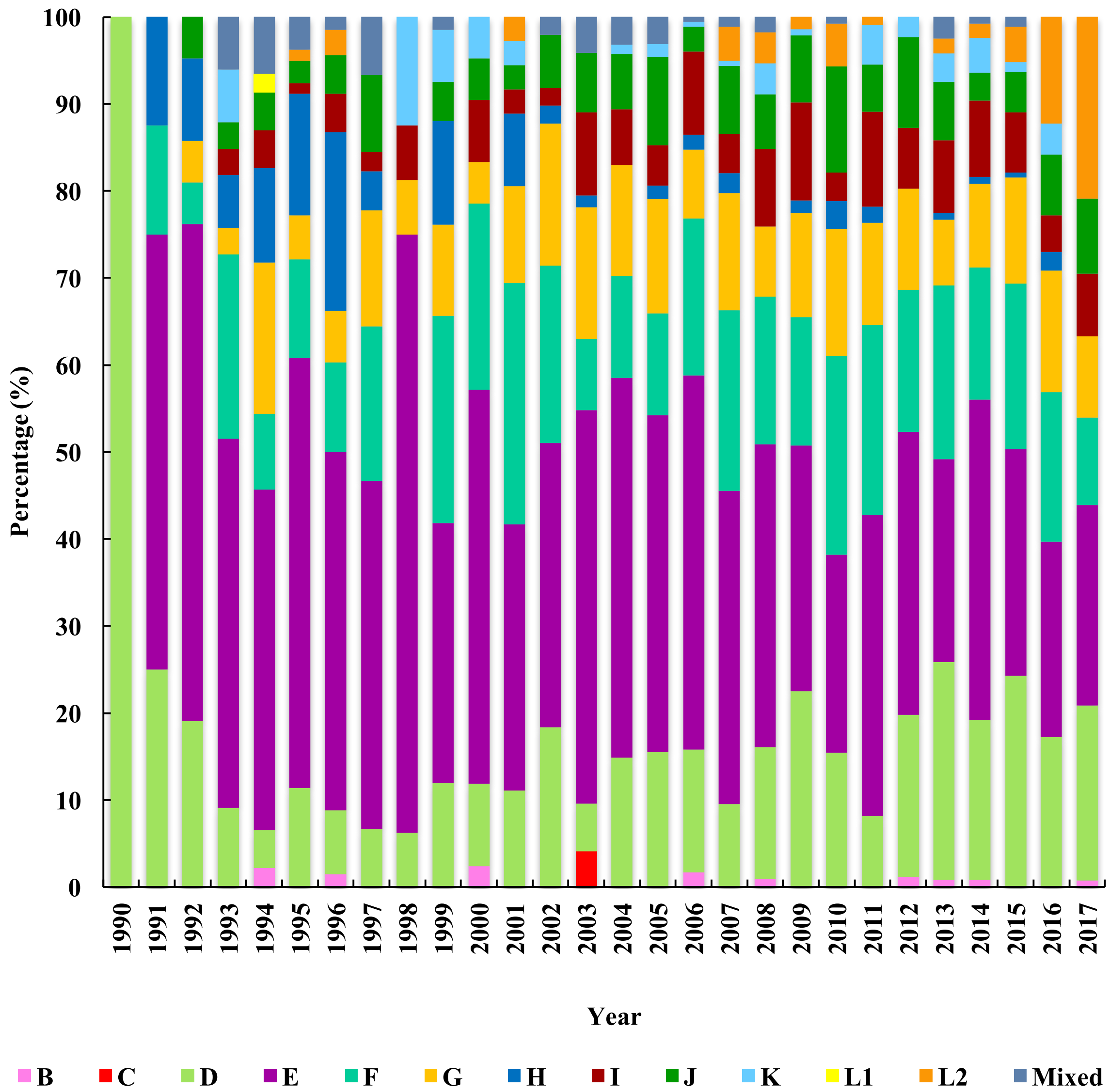


Figure 3.13 – Distribution of *ompA*-genotypes per year.

2.2. Analysis of *ompA*-genotype variants

Figure 3.14 exhibits the proportion of strains with *ompA* sequence similar to the evidenced by prototype strains versus strains with dissimilar *ompA* sequences, called as variants, among each *C. trachomatis ompA*-genotype (see also **Annex 12**). For C and I strains only variants were detected, which were quite frequent among G (217/254, 85.4%) and H (41/46, 89.1%) strains, while in genotypes E, F and K prototype strains prevail, 694/716 (96.9%), 361/370 (97.6%) and 38/39 (97.4%), respectively, despite the emergence of variants over the years for E and F.

A variant of *ompA*-genotype D, Da/IU1554, was the first D strain included in INSA's collection in 1990. D prototype strain wasn't detected until 1993 and along with the variants Da/IU1554 and Da/CS908/07 constitute the most frequently detected D genotypes.

Also for *ompA*-genotype G, the oldest strain in the collection was a variant which became predominant since 2005; however, the prototype G strain is also commonly detected.

For *ompA*-genotype I, the Ia/IU4168 variant is the most frequent followed by Ia/CS190/96.

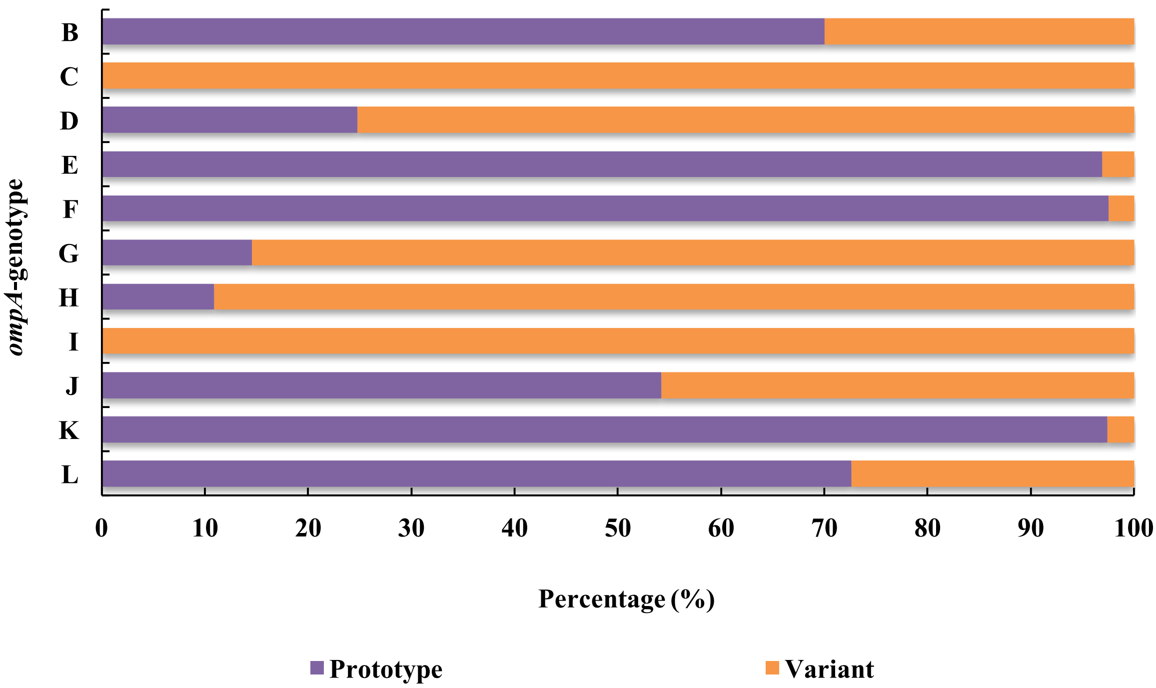


Figure 3.14 – Proportion of *ompA* prototype and variant strains among INSA's *C. trachomatis* collection.

For *ompA*-genotype J, prototype strain was slightly more frequent than variants J/CS820/07 and Ja/IUA795.

At INSA's collection, all strains identified as belonging to *ompA*-genotype K were similar to the prototype, until 2016, when the first and only variant was detected, to date.

ompA-genotype L2 strains were mostly similar to the prototype until recent years when some variants occurred, namely L2/L2b.

Table 3.1 lists identified *ompA* varieties within same *ompA*-genotype strains along the years.

Table 3.1 –Nucleotide sequence variation within *ompA* for each genotype in relation with the respective prototype strain (Table based in *Nunes et al*, 2009 [41]).

ompA-genotype (no. of variants) [a]	No. of variable sites [b]	MOMP regions [c]	Nucleotide position [d]	Amino acid change [e]	No. of strains [f]
B (3)	15	CDI	154	Thr → Ala[1]	2/3
		CDI	163/164	Val → Cys[1]	3/3
		CDI	184/186	Met → Val[1]	2/3
		CDI	195	Met → Val[1]	2/3
		CDI	198	Met → Val[1]	2/3
		CDI	216	Met → Val[1]	3/3
		CDI	228	Met → Val[1]	2/3
		CDI	246	Met → Val[1]	2/3
		CDI	249	Met → Val[1]	2/3
		VDI	268	Thr → Ala[1]	2/3
		VDI	286/287	Val → Thr[1]	2/3
		VDII	514	Ala → Thr[1]	3/3
C (3)	5	CDI	155	Thr → Ile[1]	1/3
		CDI	183	Thr → Ile[1]	1/3
		VDI	284	Gly → Asp[1]	1/3
		VDIII	693	Gly → Asp[1]	1/3
		VDIV	1003	Ala → Ser[1]	3/3
D (279)	17	CDI	154	Ala → Thr	259/279
		CDI	184/186	Val→ Met	257/279
		CDI	195	Tyr → Tyr	257/279
		CDI	223	Lys → Glu	257/279
		CDI	228	Thr → Thr	257/279
		CDI	246	Phe → Phe	257/279
		CDI	249	Gln → Hys	257/279
		VDI	286	Ala → Thr	1/279
		CDII	436	Leu → Phe[3]	3/279
		VDII	485	Asn → Ser[1]	19/279
		VDII	488	Glu → Gly[1]	1/279
		CDIII	636	Asn → Ser[1]	147/279
		VDIV	976	Ala → Thr[1]	39/279
		VDIV	977	Ala → Val	1/279
		VDIV	998	Ala → Val[1]	1 /279

		CDV	1050	Lys → Lys[1]	1/279
E (22)	12	CDI	183	Arg → Arg	2/22
		CDI	224	Lys → Arg	1/22
		VDI	286	Thr → Ala	1/22
		CDII	420	Gly → Gly	2/22
		VDII	486	Asn → Asn	1/22
		VDII	512	Asn → Ser[1]	1/22
		VDII	514	Ser → Ala[1]	5/22
		VDII	517	Val → Leu	1/22
		CDIII	568	Ala → Thr[1]	2/22
		VDIV	933	Thr → Thr	1/22
		VDIV	995	Ser → Asn[1]	1/22
		VDIV	997	Ala → Thr[1]	4/22
F (9)	9	CDI	167	Asp → Gly[1]	1/9
		CDI	180	Met → Ile[1]	1/9
		VDI	293	Thr → Ile	1/9
		CDII	388	Ile → Val[1]	1/9
		CDIII	703	Phe → Leu[1]	1/9
		VDIII	773	Ala → Val[3]	1/9
		CDIV	780	Thr → Thr	1/9
		CDIV	802	Asp → Asn[1]	1/9
		VDIV	1003	Thr → Ala[3]	1/9
G (217)	11	CDI	228	Thr→ Thr[1]	38/217
		CDII	374	Cys → Tyr[1]	2/217
		VDII	487	Gly → Ser[1]	67/217
		CDIII	566	Asp → Gly	1/217
		CDIII	700	Glu → Gln[1]	38/217
		CDIV	857	Thr → Ile[1]	1/217
		CDIV	921	Gln → Gln[3]	4/217
		VDIV	985	Ser → Gly[2]	1/217
		VDIV	1003	Ser → Ala[1]	144/217
		VDIV	1003	Ser → Thr[1]	40/217
		VDIV	1015	Ile → Val[1]	1/217
H (41)	7	VDI	272	Asn → Ser[1]	41/41
		CDII	327	Pro → Pro[1]	2/41
		CDII	383	Ala → Val[1]	1/41
		VDII	507	Asp → Glu	1/41
		VDII	514	Thr → Ala	1/41
		CDIV	810	Asp → Asp	1/41
		CDIV	850	Leu → Leu[1]	41/41
I (152)	16	VDI	277	Val → Ile[1]	148/152
		VDII	526	Val → Ile[1]	80/152
		CDIII	573	Asp → Asp	1/152
		CDIII	577	Thr → Ala[1]	1/152
		CDIII	684	Leu → Leu	150/152
		VDIII	764	Ile → Thr[1]	151/152

		CDIV	840	Leu → Leu[1]	150/152
		CDIV	932	Lys → Arg[1]	2/152
		VDIV	986	Lys → Arg	2/152
		VDIV	1000	Ser → Ala	150/152
		VDIV	1007	Ala → Gly	150/152
		VDIV	1009	Ser	150/152
		VDIV	1014	Glu → Asp	150/152
		VDIV	1020	Glu → Asp	150/152
		CDV	1119	Asp → Asp	141/152
		CDV	1146	Arg → Arg	141/152
J (71)	25	CDI	195	Tyr → Tyr	1/25
		CDI	198	Gly → Gly	1/25
		CDI	213	Asp → Asp[1]	2/25
		VDI	268/269	Thr → Val	31/25
		VDI	310	Val → Ile	31/25
		CDII	369	Asn → Asn	37/25
		VDII	499	Ala → Ser	31/25
		VDII	522	Lys → Asn	31/25
		VDII	526	Phe → Ile	31/25
		VDII	544	Asn → Asp[1]	2/25
		CDIII	555	Val → Val	31/25
		CDIII	633	Thr → Thr	1/25
		CDIII	681	Glu → Glu	31/25
		CDIII	684	Leu → Leu	33/25
		CDIII	720	Asn → Asn[1]	2/25
		VDIII	742	Ala → Thr[1]	2/25
		VDIII	753	Pro → Pro[1]	2/25
		VDIII	757	Asp → Asn	1/25
		VDIII	782	Ala → Glu[1]	1/25
		CDIV	813	Tyr → Tyr	31/25
		VDIV	991	Thr → Ala[1]	2/25
		VDIV	997	Val → Ile	31/25
		VDIV	1000	Ala → Ser[1]	2/25
		VDIV	1020	Asp → Glu[1]	33/25
K (1)	1	VDIV	972	Pro → Pro	1/1
L2 (26)	5	VDII	485	Asn → Ser[1]	25/26
		VDII	494	His → Arg[3]	1/26
		VDII	515	Lys → Thr[3]	1/26
		VDII	517	Leu → Phe[1]	1/26
		VDII	517	Leu → Ile[3]	3/26

[a] ***ompA*-genotype (number of variants) –** *ompA*-genotypes with the number of variants found in the database;

[b] **Number of variable sites –** Number of variable sites found in the database;

[c] **MOMP region –** Indicates the region where the variable sites are located; CD – conserved domain; VD – variable domain; for both, I, II, III or IV;

[d] **Nucleotide position –** based on the alignment of the strains of the same genotype;

[e] **Amino acid change –** comparison of the variant with the prototype for each *ompA*-genotype. Ala – Alanine, Arg – Arginine, Asn – Asparagine, Asp – Aspartic acid, Gln – Glutamine, Glu – Glutamic acid, Gly – Glycine, His – Histidine, Ile – Isoleucine, Leu – Leucine, Lys – Lysine, Phe – Phenylalanine, Ser – Serine, Thr – Threonine, Val – Valine. [1] Described in *Nunes et al*, 2009 **[41]**, [2] Described by *Li, C. L. et al.* The molecular epidemiology of genital *Chlamydia trachomatis* in China. Unpublished. 2005, [3] Described by *Caldeira, A. R. S. V.* **[166]**
[f] **Number of strains –** number of strains sharing each variable site.

A total of 123 nucleotide-variable sites were identified, with genotypes J, D and I presenting the highest number of sites (25, 17 and 16 changes, respectively), while genotype K was the least variable, with only one change.

Each variable site was represented by only two types of nucleotides, with the exception of *ompA*-genotype G and L2, which present a parsimony-informative site with two different non-synonymous changes found for the same variable site at positions 1003 and 517, respectively. Also, some of the *ompA*-genotype I exhibit an addition of a Serine in position 1009.

Overall, for the identified variable sites, 62/123 (50.4%) occurred in VDs and 61 (49.6%) in CDs, with CDI encompassing for 47.5% (29/61) of them.

Mutations were more frequently detected in VDIV, followed by the VDII and the CDIII. Variable sites on VDI only represent 6.3%, and on VDIII, only 1.0% (**Figure 3.15**).

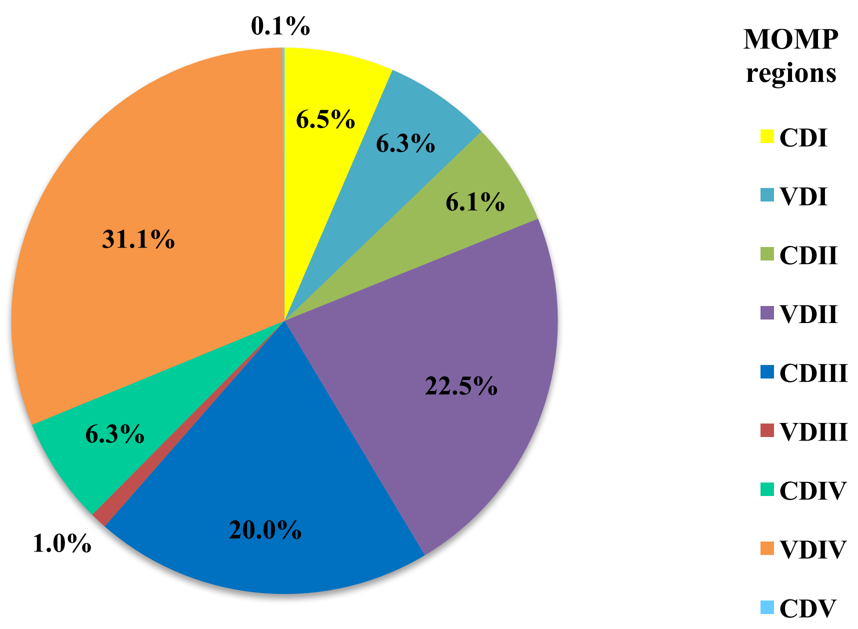


Figure 3.15 – Frequency (in %) of variable sites per CD and VD of the *ompA* gene.

3. Evaluation of intra-patient *C. trachomatis* genetic heterogeneity affecting homopolymeric tracts potentially driving phase variation, among *C. trachomatis*-positive DNA samples selected from the collection of the Portuguese National Institute of Health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, I.P.)

In previous studies [60, 166], we were able to unveil potential mechanisms of phase variation in *C. trachomatis* mediated by in-length variable homopolymeric tracts (poly (Ns)). Here, we scaled-up the analyses for 171 *C. trachomatis*-positive samples (**Annex 4**), which were scrutinized regarding the intra-patient variability of 12 selected potentially variable poly (N). By exploring their intra-host genetic diversity, we ultimately aimed at unveiling novel phase variation mechanisms in *C. trachomatis*.

Of note, for this particular sample set, strains from the genital area are the most variable according to the *ompA*-genotypes found, as shown in **Figure 3.16**, followed by the rectum specimens.

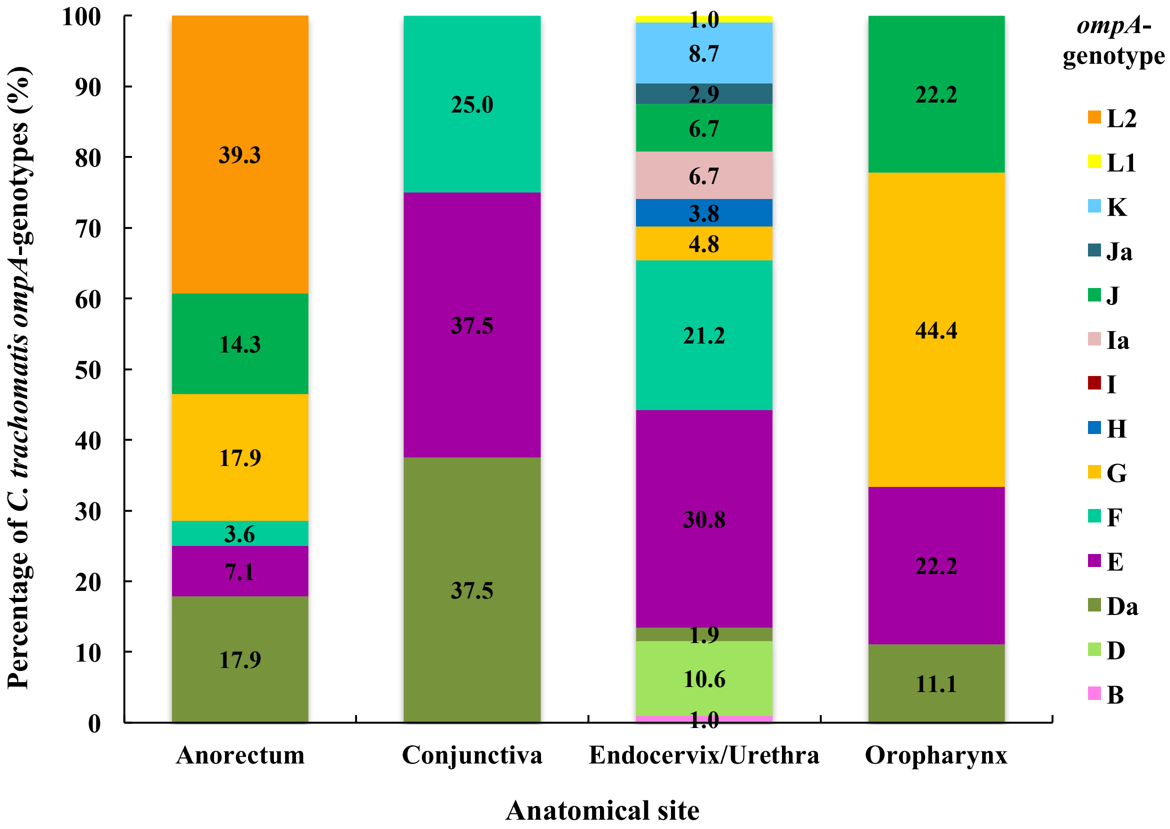


Figure 3.16 – Distribution of *C. trachomatis ompA*-genotypes per anatomical site.

For each poly (N), we correlated the observed intra- and inter-patient variability with the genetic clade (ocular, LGV, T1 or T2) of the *C. trachomatis* strains. Giving that the large majority of the available clinical specimens were collected from the genitalia and limited data was available regarding clinical outcomes, no association could be established with the anatomical site of infection, patient gender or disease severity. For the samples described by Hadfield, J. et al., 2017 [48], as they have been subjected to deep genome and phylogenetic characterization, the genetic clade was already known. For the remaining samples, we inferred the genetic clade after the identification of *bona fide* clade-specific genetic markers. These were found after the inspection of a previously generated whole-genome alignment of well characterized strains [60] for genome positions whose mutational profile perfectly segregated a given clade (BLAST analyses subsequently confirmed the specificity). Within the genome regions covered by amplicons, we identified 11 markers specific of clade T1 and 4 markers perfectly segregating LGV strains (strains were classified as T2, if they displayed an urogenital *ompA*-genotype and did not possess any of the above referred nucleotide markers; strains with ocular *ompA*-genotypes were assumed to fall into the ocular phylogenetic clade) (**Table 3.2**). **Annex 4** lists the known/inferred genetic clade for each strain.

Table 3.2 – Genetic markers clade-specific for T1 and LGV strains.

Genetic Marker	Genome Position *	Loci	Clade-specific Base	Clade
1	48003	upstream CT042/glgX	A	T1
2	48095	upstream CT042/glgX	C	T1
3	291641	CT259	A	T1
4	291876	CT259	G	T1
5	367345	CT326.1	C	T1
6	516300	upstream CT445/gltX	A	T1
7	516348	upstream CT445/gltX	T	T1
8	516386	upstream CT445/gltX	A	T1
9	516438	upstream CT445/gltX	G	T1
10	607633	upstream CT541/mip	11bp del	T1
11	967067	CT823/htrA	T	T1
12	195622	CT172.1	A	LGV
13	367258	CT326.1	C	LGV
14	1031888	CT871/pmpG	A	LGV
15	1032011	CT871/pmpG	A	LGV

* Position regarding the D/UW3 genome annotation (accession number NC_000117).

All selected poly (N) revealed intra-patient variability for at least one sample, where four of them (CT259, CT871, CT445 and CT561) showed variation in more than 50% of the samples for which data could be collected (**Table 3.3**). Remarkably, the poly (G) tract affecting the functionality of a well-known *C. trachomatis* cytotoxin (CT166), which were previously hypothesized to be regulated by phase variation, also revealed high variability, not only at intra-patient level (i.e., presence of clones within an *in vivo* population carrying dissimilar lengths of nucleotide repeats), but also at inter-patient level (i.e., the dominant profile varies between patients).

Table 3.3 – Ranking of poly (N) by the proportion of samples displaying intra-patient variability.

Name	Percentage of samples with intra-patient variability*	n
polyCT259	84.8%	67/79
polyCT871	62.3%	91/146
polyCT445	58.6%	58/99
polyCT561	53.6%	59/110
polyCT166	46.0%	40/87
polyCT605	38.8%	59/152
polyCT172	28.3%	39/138
polyCT326	19.4%	28/144
polyCT541	18.6%	29/156
polyCT823	13.9%	15/108
polyCT694	11.2%	15/134
polyCT042	3.1%	4/131

* Intra-patient variability is considered when, in a given sample, dominant 'count' represented less than 90% of all respective reads (Forward + Reverse) counted in that region.

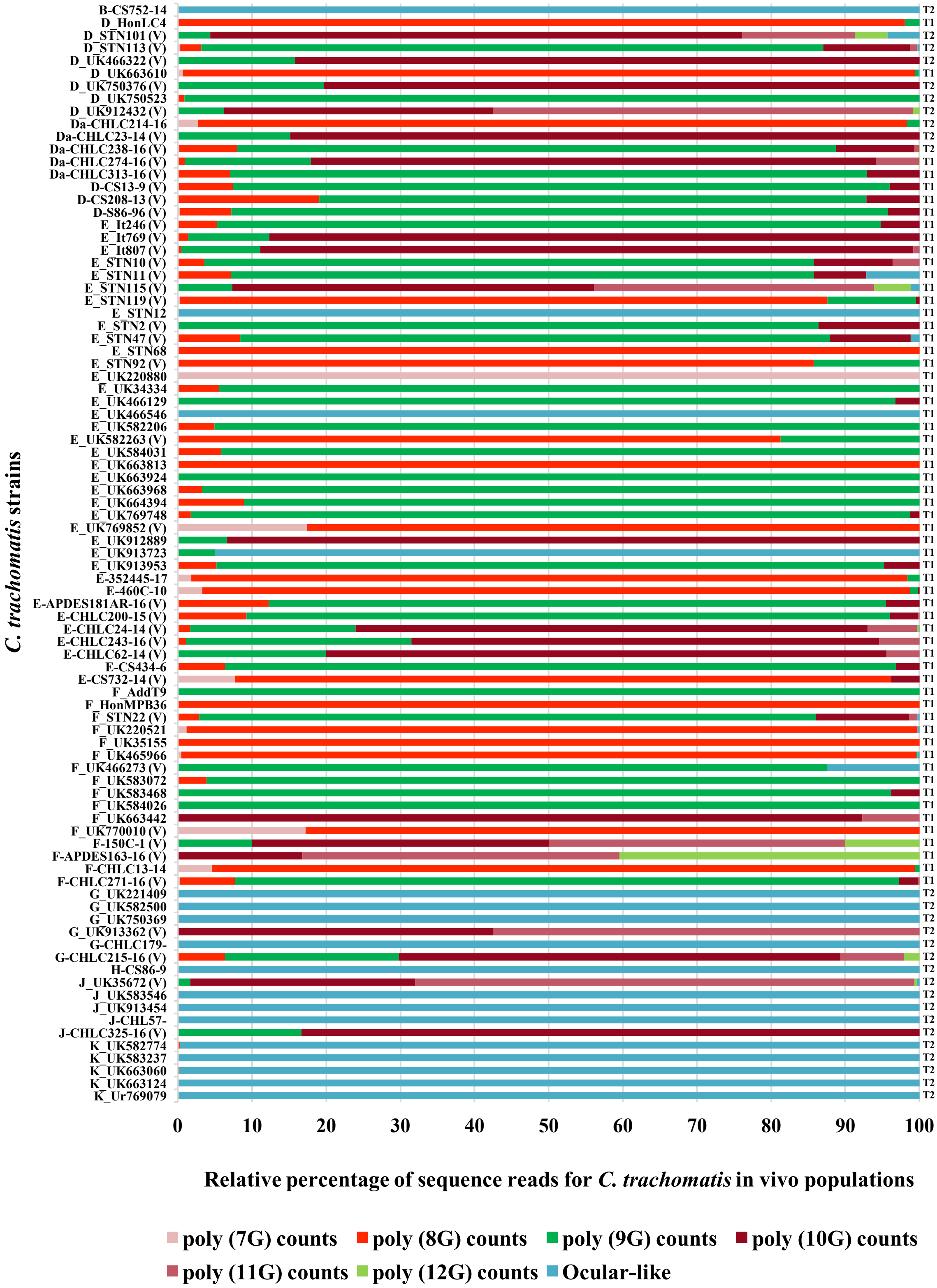


Figure 3.17 – Genetic heterogeneity in the homopolymeric tracts, probably driving phase variation of the virulence factor CT166 (*C. trachomatis* cytotoxin). Each bar displays the relative percentage of sequence reads with a particular base count for 87 *C. trachomatis in vivo* populations. Profiles in red color range represent poly (G) count potentially yielding protein truncation (OFF), while green color range represent a functional protein (ON). The color blue represents a profile typically observed in ocular strains ("ocular-like"), with an interruption of the poly (G) by a nucleotide A (GGAGGGGG). Only base counts relying on a count coverage >10 were considered. Profiles were marked as 'variable' **(V)** in a given sample if the dominant 'count' represented less than 90% of all respective reads (Forward + Reverse) counted in that region.

The results for the cytotoxin gene (CT166) markedly evidenced intra-strain variability, which was similar between both clades T1 and T2. In general, depending on base-count, the cytotoxin protein is expected to be functional (ON) or truncated (OFF) and, more than a third of the strains included in the study evidenced a predicted functional protein (35.6%, n=31), i.e., the dominant profile is poly (9G) or poly (12G) (**Figures 3.17** and **3.18**). Importantly, for clade T1, the majority of strains (n=31) had a truncated protein (mostly mediated by poly (8G) and poly (10G)) and for clade T2, most strains had an ocular-like profile (n=14), also yielding a non-functional protein **(Figure 3.18**). Of note, the CT166 gene is absent in LGV strains [51].

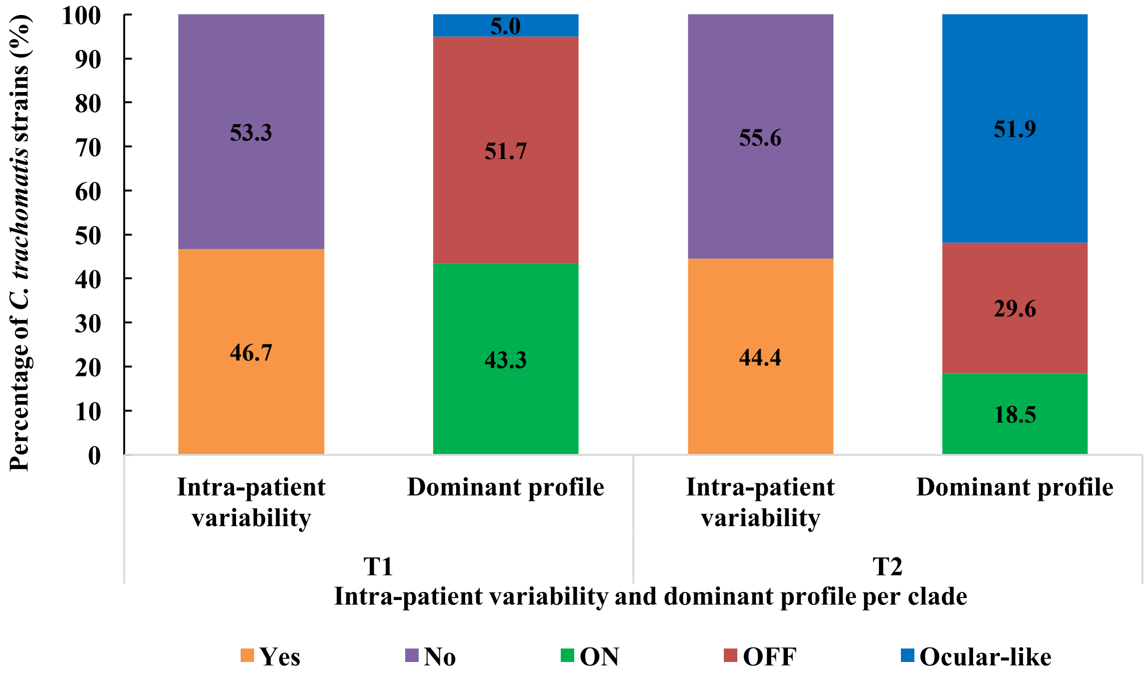


Figure 3.18 – Intra-patient variability and profile of the homopolymeric tracts affecting CT166 in *C. trachomatis* strains, according to clade (see also **Annex 13 a)**).

The poly (Ns) potentially affecting CT042 and CT694, both located in their possible regulatory region, revealed vestigial intra-patient variability, as shown in **Figures 3.19** and **3.20**, with only very few strains revealing variability. Still, the dominant profile displayed some clade-specificity: the poly (8G) was mainly found in LGV stains for CT694 and a particular profile GAGGGG was exclusively observed for T2 isolates for CT042.

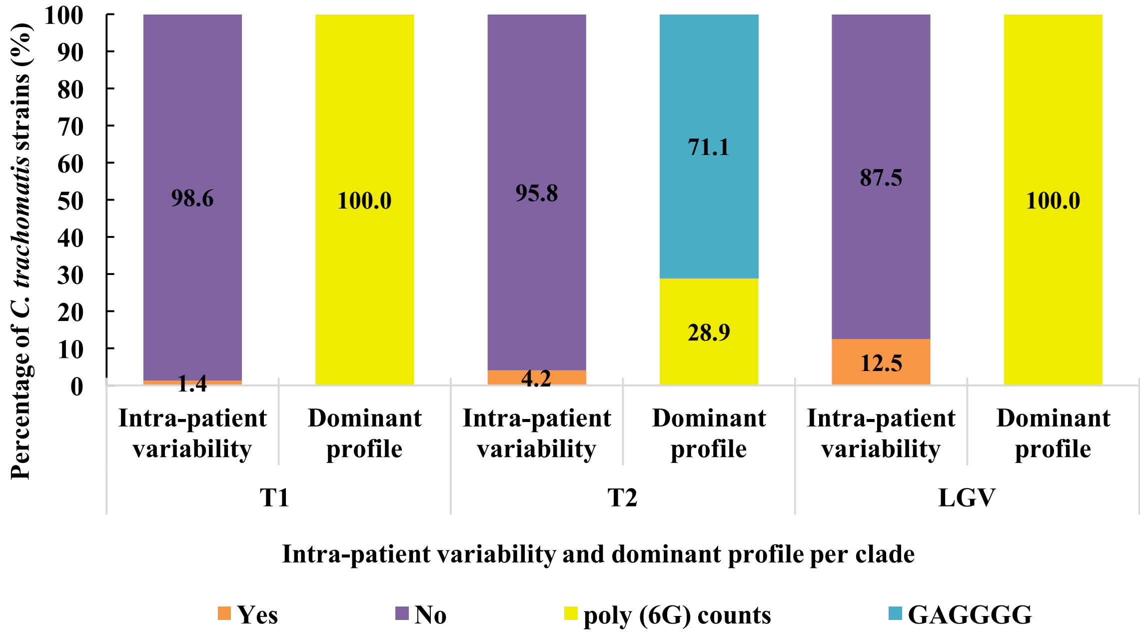


Figure 3.19 – Intra-patient variability and profile of the homopolymeric tracts affecting CT042 in *C. trachomatis* strains, according to clade (see also **Annex 13 b)**).

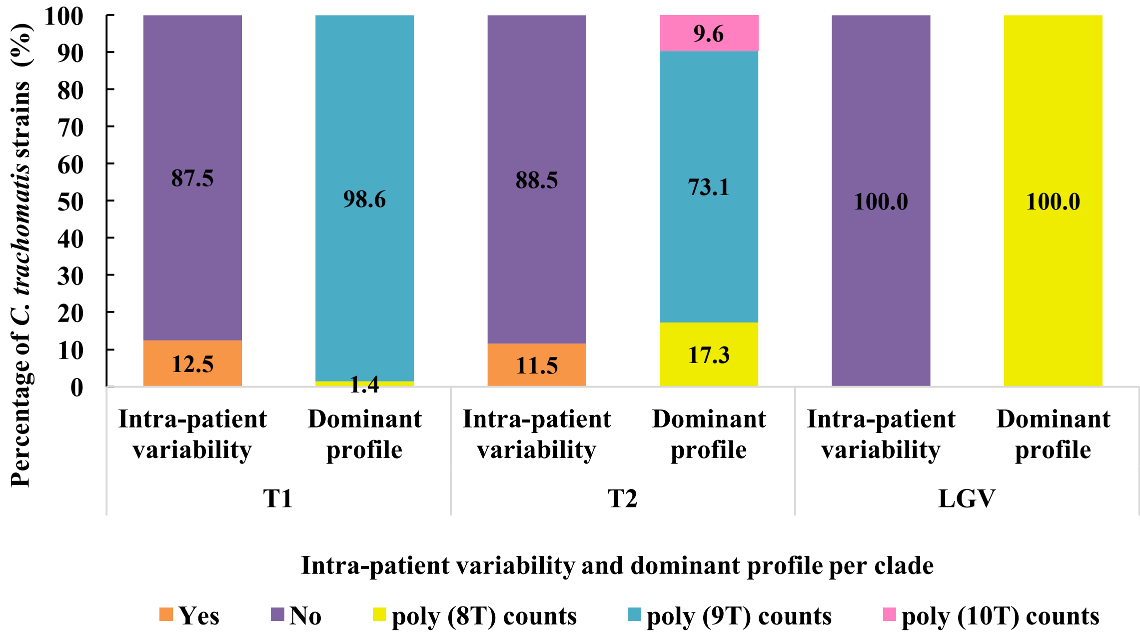


Figure 3.20 – Intra-patient variability and profile of the homopolymeric tracts affecting CT694 in *C. trachomatis* strains, according to clade (see also **Annex 13 c)**).

Regarding the results obtained for the homopolymeric tracts located in a possible regulatory region affecting CT561 and CT605 (**Figures 3.21** and **3.22**, respectively), we could observe that clade T1 strains displayed highly intra-patient variability for both poly (Ns) (the degree of intra-strain variability for CT561 was the highest reported). In contrast, these poly (Ns) revealed little variability within strains from other groups. It is also worth noting that both poly (Ns) revealed a different dominant profile for each clade, with exception of the clade T2 strain for CT561, for which we found strains with poly (9A) counts but also with poly (8A) counts.

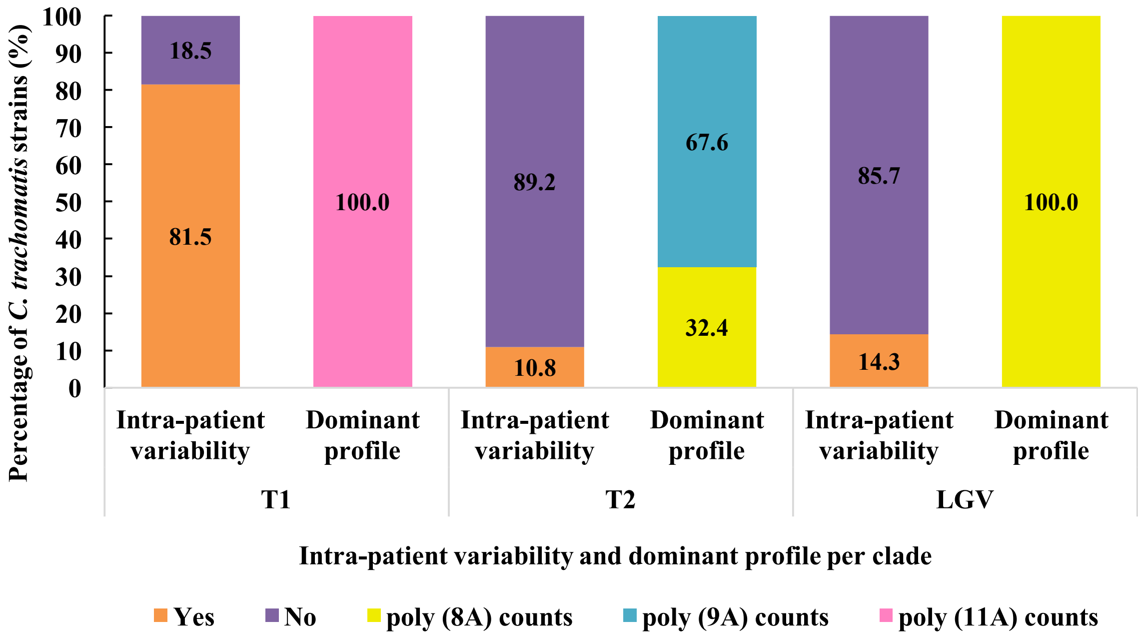


Figure 3.21 – Intra-patient variability and profile of the homopolymeric tracts affecting CT561 in *C. trachomatis* strains, according to clade (see also **Annex 13 d)**).

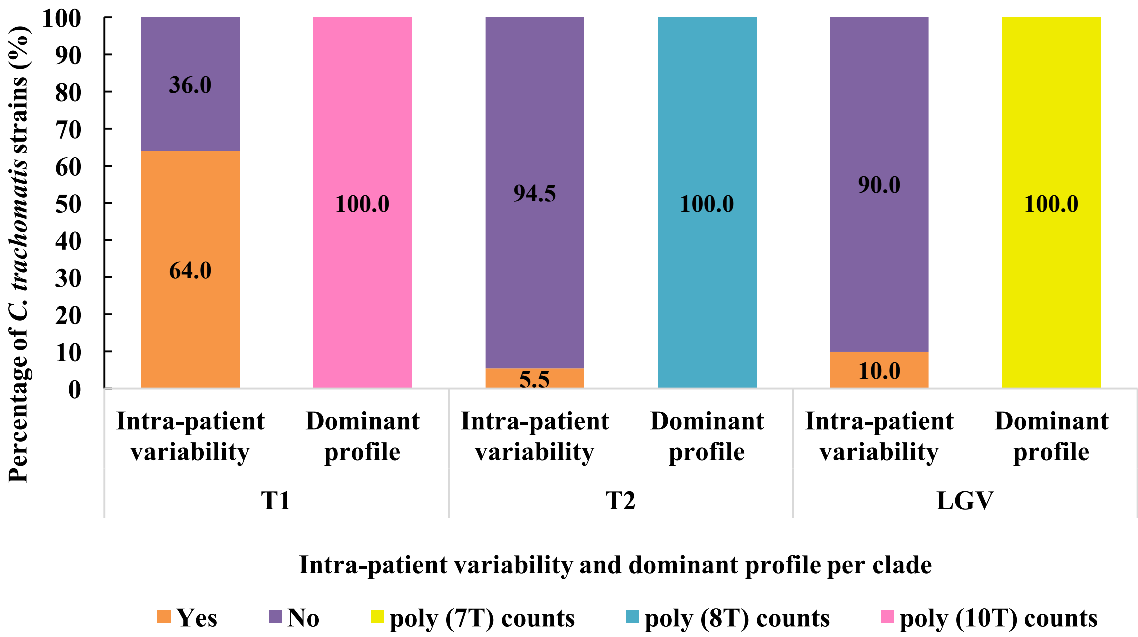


Figure 3.22 – Intra-patient variability and profile of the homopolymeric tracts affecting CT605 in *C. trachomatis* strains, according to clade (see also **Annex 13 e)**).

Figures 3.23 and **3.24** represent the results achieved for the homopolymeric tracts affecting the possible regulatory region of CT541 and CT172, respectively. Regarding CT541, we found intra-patient variability mostly associated with clade T2, where there was an almost equal proportion when referring to dominant profiles observed (poly (9A) and poly (10A) (n=30, n=29, respectively)). On the other hand, for CT172, the most dominant profile was found to be different for each group: LGV group had a poly (8C) dominant profile; clade T2 revealed dominant profiles with poly (9C) and poly (10C); and, clade T1 displayed as main profile a poly (6C) tract (n=52). Curiously, when looking at *ompA*-genotype level, we were able to observe that most of the strains revealing a dominant poly (6C) profile belonged to *ompA*-genotype E, while the majority of the remaining *ompA*-genotypes – Da, F and Ja – had dominant poly (8C) counts (**Figure 3.25**).

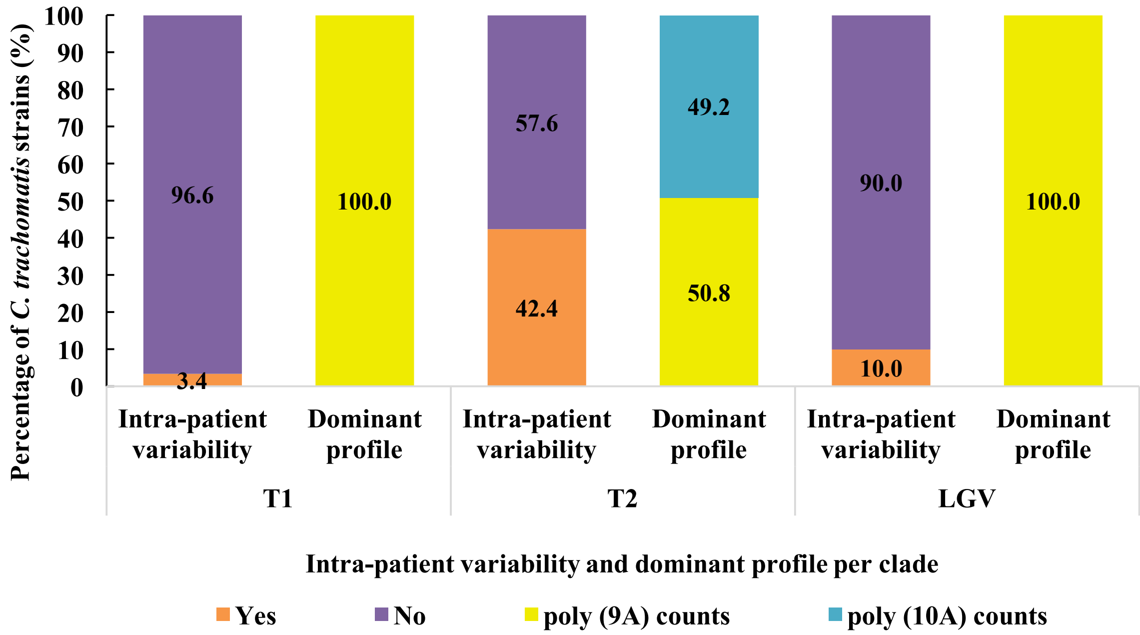


Figure 3.23 – Intra-patient variability and profile of the homopolymeric tracts affecting CT541 in *C. trachomatis* strains, according to clade (see also **Annex 13 f)**).

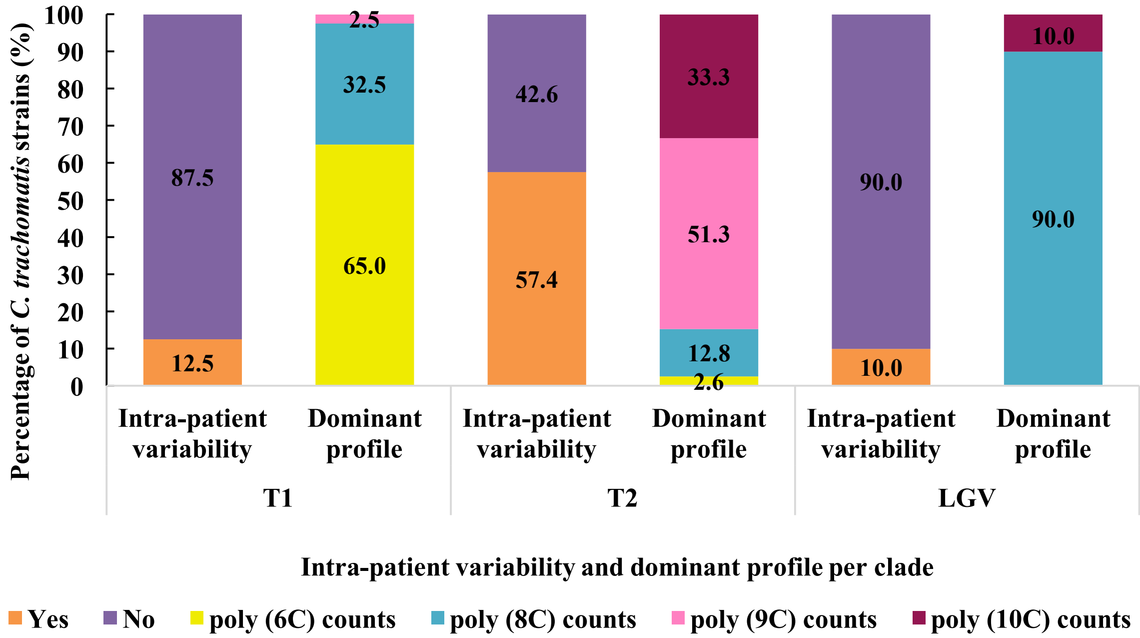


Figure 3.24 – Intra-patient variability and profile of the homopolymeric tracts affecting CT172 in *C. trachomatis* strains, according to clade (see also **Annex 13 g)**).

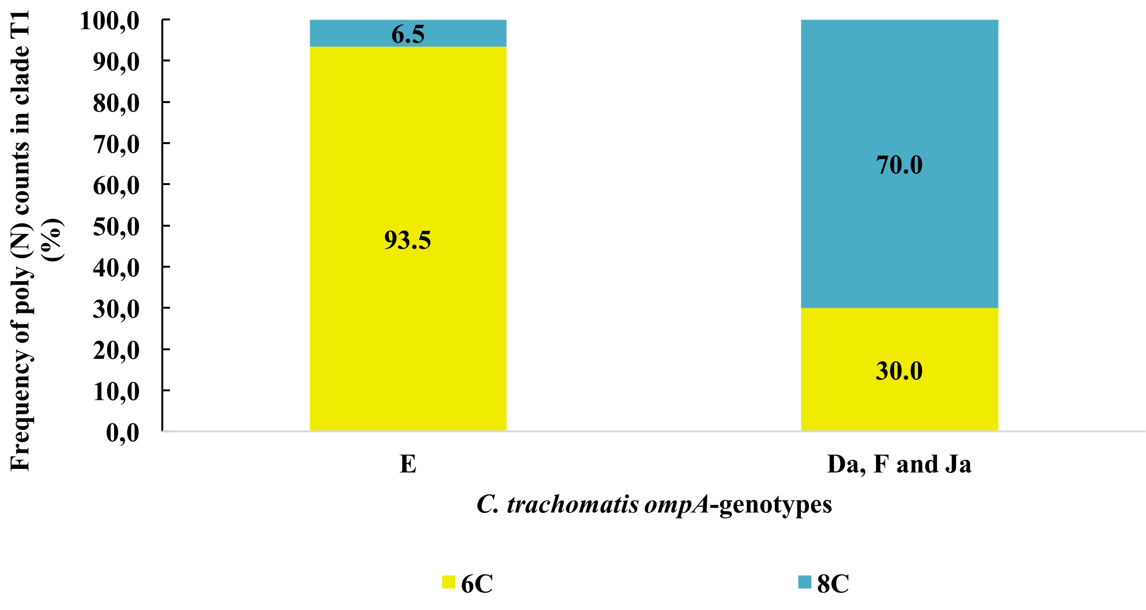


Figure 3.25 – Profile of homopolymeric tracts affecting CT172 in *C. trachomatis* strains clade T1, according to *ompA*-genotype.

For homopolymeric tracts potentially affecting CT445 and CT871 **(Figures 3.26** and **3.27**, correspondingly), we could observe that they revealed high intra-strain variability among clade T1, while for clade T2, variability occurred in almost a third of the strains (n=11, 31.4%) for CT445 gene, and in more than two thirds of the strains for CT871 gene (n=40, 72.7%). On the other hand, no variability was observed for LGV strains. Of note, the dominant profile variation revealed a group-specific profile for the homopolymeric tracts affecting the possible regulatory region of CT445, while, for coding region CT871, all T1 and T2 clades shared the same dominant profile (poly (9G) counts), which differed from the one found for all LGV strains (poly (8G) counts).

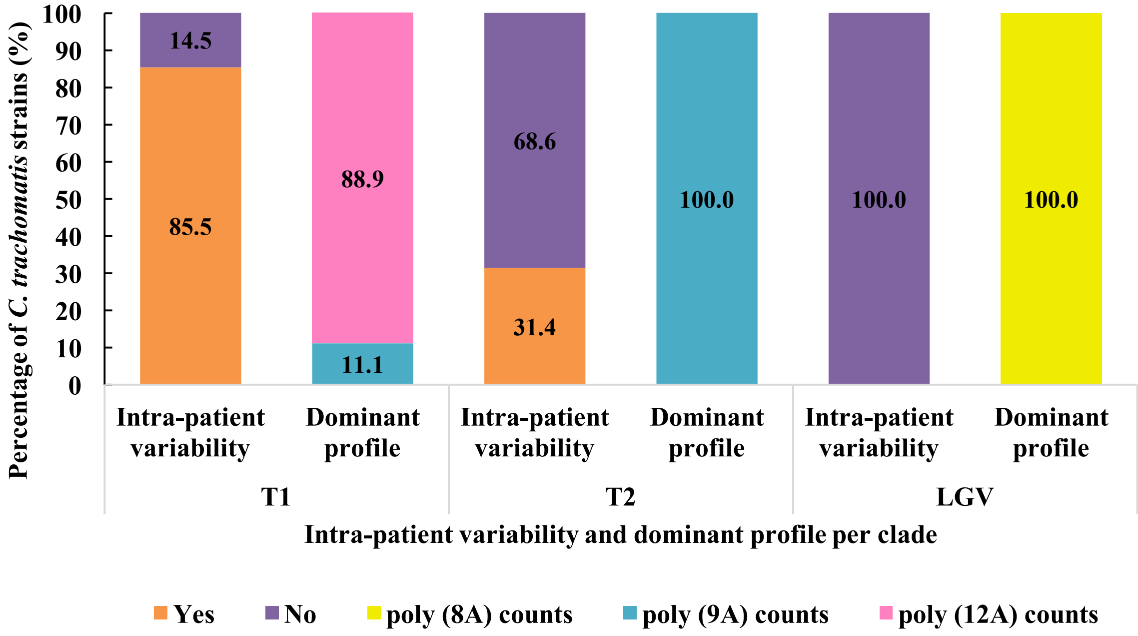


Figure 3.26 – Intra-patient variability and profile of the homopolymeric tracts affecting CT445 in *C. trachomatis* strains, according to clade (see also **Annex 13 h)**).

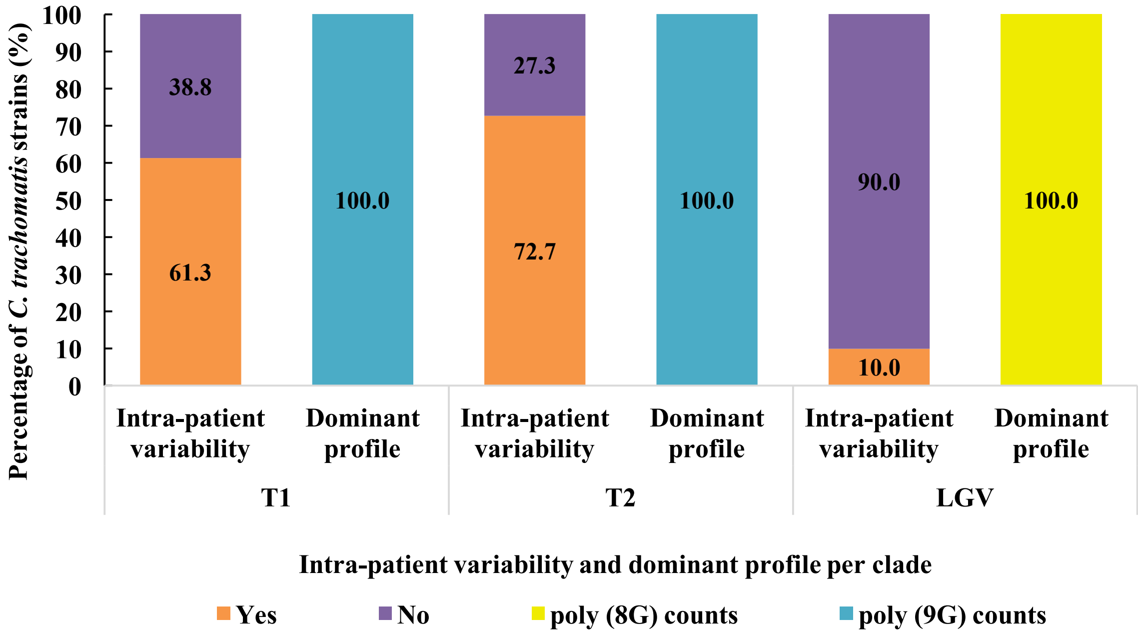


Figure 3.27 – Intra-patient variability and profile of the homopolymeric tracts affecting CT871 in *C. trachomatis* strains, according to clade (see also **Annex 13 i)**).

Results obtained for homopolymeric tracts likely affecting CT326 – situated in its coding region – and for CT823 – located in its possible regulatory region – revealed that intra-patient variability was very low for both clades T1 and T2 but, on the contrary, very high for the LGV group. Also, it is worth noting that the dominant profiles differed between clades for both poly(N)s., **(Figures 3.28** and **3.29)**.

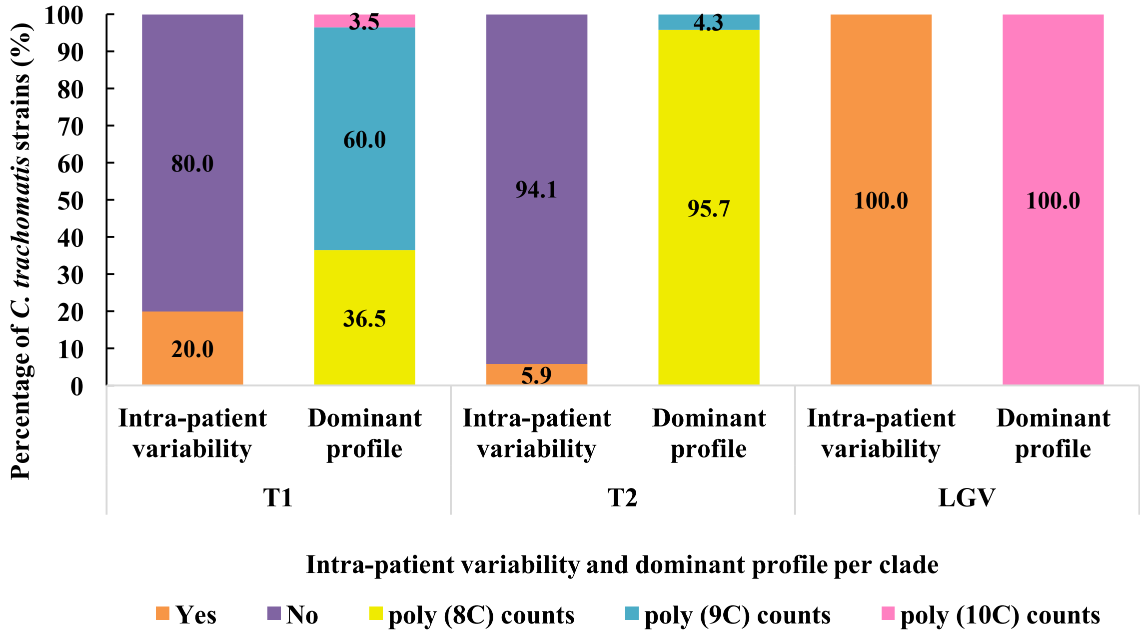


Figure 3.28 – Intra-patient variability and profile of the homopolymeric tracts affecting CT326 in *C. trachomatis* strains, according to clade (see also **Annex 13 j)**).

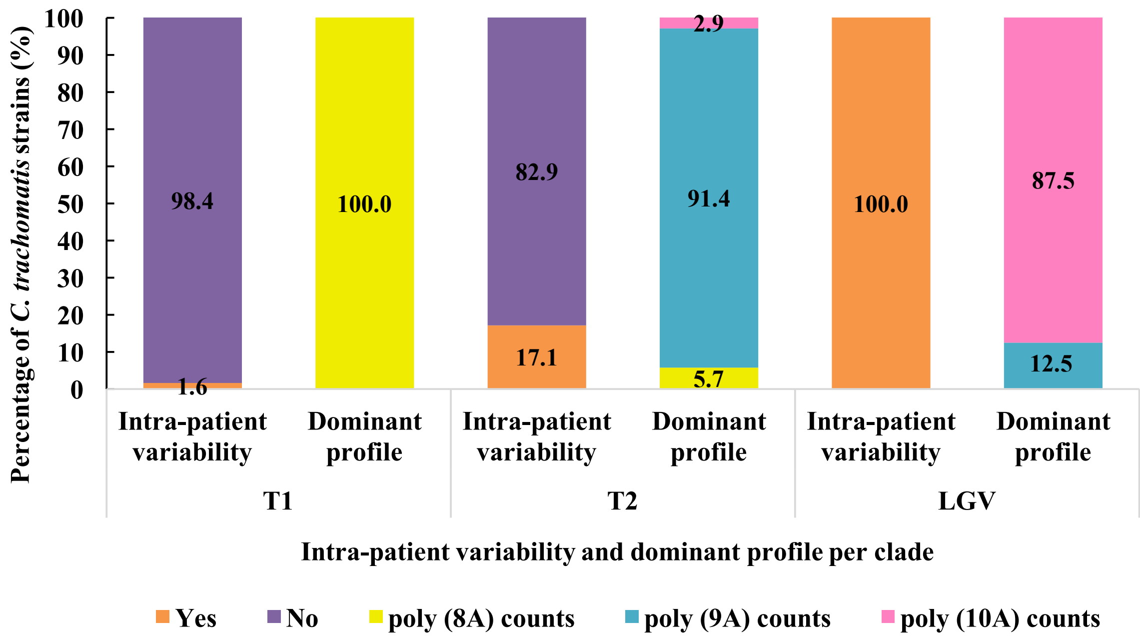


Figure 3.29 – Intra-patient variability and profile of the homopolymeric tracts affecting CT823 in *C. trachomatis* strains, according to clade (see also **Annex 13 k)**).

Finally, for the homopolymeric tract upstream from CT259, intra-patient variability was found for most of the samples of both clades T1 and T2. For instance, 53 out of the 54 samples revealed intra-patient variability for clade T1 (**Figure 3.30**); however, no data could be generated for this poly (N) for any LGV strain.

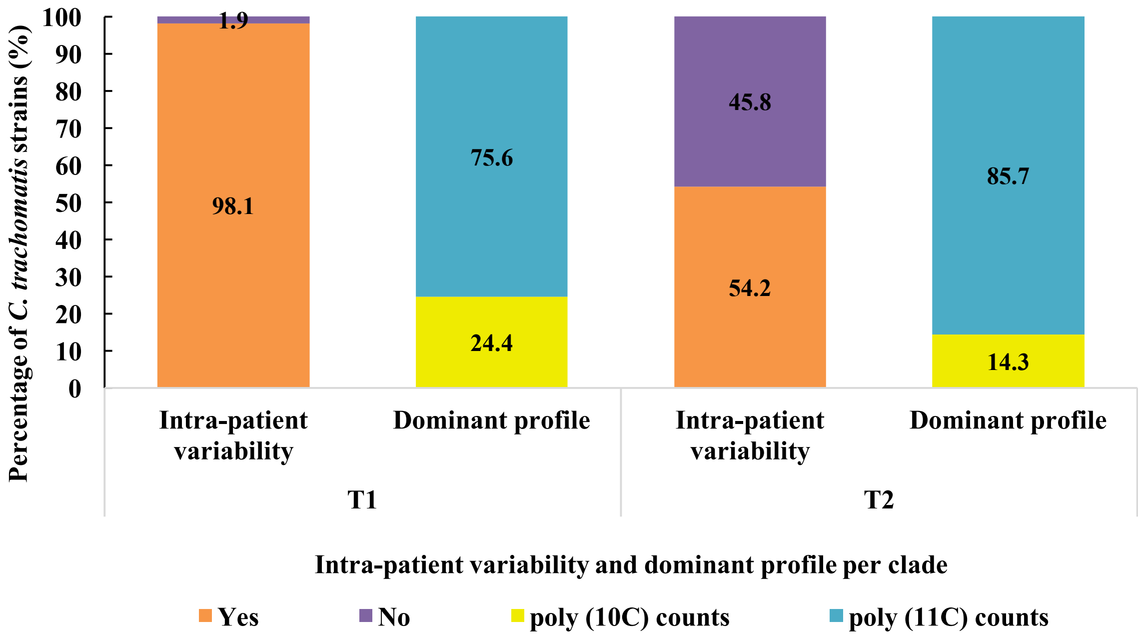


Figure 3.30 – Intra-patient variability and profile of the homopolymeric tracts affecting CT259 in *C. trachomatis* strains, according to clade (see also **Annex 13 l)**).

Discussion

1. Determination of the prevalence of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis* in patients of the major Portuguese STD clinic (Unidade de consulta de DST/CAD da Lapa)

The 'Unidade de consulta de DST/CAD da Lapa' is in the active since the year of 1987 and it is the only STI clinic in Primary Healthcare facilities in the country. Diagnosis and counseling for the prevention of STIs are objectives of this open and freely available clinic, with no limitations related to residence area, nationality or legal status. This clinic and the national reference laboratory for STIs at INSA have been collaborating from that date.

Urethral samples, namely urines, were the most common type of samples because they are non-invasive and it is easy to obtain a self-collected sample, providing good results in terms of sensitivity, with 85 to 95% for men and 80 to 90% for women [167]. First-void urine is highly recommended for detecting by NAATs, due to a higher bacterial load in comparison to the second and third portions, and because it is effective in detecting nucleic acids of microorganisms even in asymptomatic infections or at early phase of infection [168, 169]. Our study used Cobas CT-NG (Roche™), a recognized test for *C. trachomatis* and *N. gonorrhoeae* in terms of sensitivity and specificity [170, 171] and a new molecular test, S-DiaMGTV™ (Diagenode) [172]. In comparison to Cobas, this test is not automated and thus, the risks of operator contamination or exchange of samples cannot be excluded; however, it has the great advantage of using Cobas DNA eluates, for which the exact location in the eluates plate is provided by the equipment, and this surely contributes to minimize operator mishandling and avoids the need of an extra step of extraction.

Anorectal swabs were exclusively performed in MSM with anal sex intercourse, and STIs were detected in 46.4% (13/28) of the samples; however, a study [68] has shown that heterosexual couples having anal intercourse should also be screened at this location. Not all STIs are able to infect the rectal mucosa but it could be an important reservoir of *C. trachomatis* and *N. gonorrhoeae*, which may explain the statistically significant association between *N. gonorrhoeae* and sexual orientation ($p=0.002$), possibly with the MSM group, as it can be more easily transmitted upon condomless anal intercourse, when compared to vaginal and oral intercourse transmission [4, 173, 174]. MSM might be more at risk of acquiring a STI due to some reported sexual behaviors, such as condomless anal intercourse (as referred above), multiple sexual partners, rougher sex, especially under the influence of drugs and alcohol [40]. However, MSM are still stigmatized in many settings, a situation that might induce people to avoid seeking diagnose and treatment.

STIs screening in the oropharynx is often neglected [175] and, in this study, only 2.3% (24/1034) of the samples were from this anatomical site. This may reflect the fact that patients hide their oral sex practices, misguiding the healthcare providers. Although a study [176] reported that one-third of the women with oropharyngeal STIs had it as the only site of infection, and that almost all cases were asymptomatic, this anatomical location remains highly under-diagnosed/neglected, in what concerns to STIs screening. Among MSM, a significant increase in detection of STIs occurs when oropharyngeal and anorectal sites are tested, in addition to the routine testing of urine/urethral samples [177, 178]. If those sites were to be tested only when clinical manifestations were present, STIs would be missed at oropharynx and anorectal sites due to asymptomatic character of these infections. Thus, the knowledge on the sexual habits of each patient, is fundamental [176]; a study showed that if the rectal site had been missed for STIs testing, 60 to 80% of the cases in MSM and over 20% of the cases in women would have been missed [173].

Although 73.9% of the attendees of the STD clinic were 25 years and older, *C. trachomatis*, *N. gonorrhoeae* and *M. genitalium* were more frequent among young attendees (**Figures 3.3**, **3.5** and **3.7**) and only *T. vaginalis* was more common in the oldest age group, which was to be expected in consonance with other studies relating *T. vaginalis* and older ages [146]. The higher frequency in younger ages was to be expected; in fact, younger people are more likely to have ectropion or everted columnar epithelium into the ectocervix, predisposing them to greater risk of acquiring a STI [179]. On the other hand, older people could be less prone to risky sexual behaviors when compared to younger people [180]. However, in our study, people with 25 years and older accounted for 63% of the STIs positives; a situation that can be explained by the lack of protective immunity. In fact, despite the development of some immunity upon suffering several STIs episodes, it doesn't provide enough protection against the acquisition of a new STI [181].

It is known that multiple partners are often associated with increased risk of acquiring and transmitting STIs [182]. In our study, more than half of patients reported having two or more sexual partners in the last six months, and 20% (123/614) of them were detected with one of the STIs under evaluation (**Figures 3.4**, **3.6** and **3.8**) and almost a tenth (8.5%, 52/614) had HIV.

Considering the reasons for attending to the STI clinic, 'screening' or 'having symptoms', frequency of the evaluated STIs was higher for the latter category (21.3%, 100/470), rather than among the 'screening' category (14.9%, 77/517). Although the four STIs under evaluation are frequently asymptomatic and considering that the screening may reduce the reservoir of infections in high prevalence communities [91], the existence of symptoms is still the most common motif associated with clinical visit and being infected. However, symptoms, when present, may be commonly nonspecific. Therefore, there should be an investment in providing education and counselling of individuals, but most of all, there should be increasing in testing, appropriate diagnosis and treatment of infected people and their partners, which is of great importance in order to reduce and prevent the spread of STIs [9].

Almost a third of our study population (n=298, 28.8%) had at least one STI in the past, and 41.6% (n=124) of the people from this group had also a STI in the present. This is of great distress since it is known that re-infections facilitate transmission and acquisition of HIV [105], among other consequences such as increasing infectivity by altering host immune defenses, increasing of the duration of the erosions and development of sequels [105]. Furthermore, constant reinfections evidence lack of prevention methods and repeated high-risk behaviors.

Almost a fifth of our study population was found to be diagnosed with at least one STI (*C. trachomatis, N. gonorrhoeae, M. genitalium* or *T. vaginalis*) and the most frequent (10.4%) was *C. trachomatis*, as expected, according to its prevalence in the USA and Europe, and because the number of detected cases have been rising the past few years [1, 7, 66]. Reported rates may underestimate the true burden of *C. trachomatis* infections due to their asymptomatic character. However, its increased frequency during the last decades may also be a consequence of the expansion of screening, namely among asymptomatic, because of the development of better and more sensitive diagnostic methods, and also due to the improvement of reporting systems [9]. Hence, there is a continuing need for healthcare providers sensibilization for these infections, their diagnosis and treatment in order to actually reduce the incidence rates in populations.

C. trachomatis infection is usually more frequent among women rather than men [7, 67]. However, in our study, we have observed the opposite. This was probably due to the type of attendees of the STI clinic, which is majorly composed by men (in our study population men were 2.5 more frequent than women). Epidemiologically, men can also act as a large *C. trachomatis* infection reservoir for the female population, sustaining the pathogen within a community.

C. trachomatis prevalence was described [69] to be higher in young adults with ≤24 years old and, in our study, a statistical significant association between chlamydial infection and the age (p=0.015) could be established. This is of great concern, especially among young adults, since non-treated infections can ascend to the upper genital tract and cause pelvic inflammatory disease and related sequelae, such as ectopic pregnancy and tubal factor infertility [38] in young women – which are preventable if early detected and treated [32].

Chlamydial infection was found to have an association with the anatomical site (p<<0.001), with endocervix/urethra as the region more often tested; *C. trachomatis* at this site occurred in both women and men (including MSM), as described in other studies [2, 10, 182]. The oropharynx was the most neglected anatomical site and therefore few positive results were detected (1/24); in fact, just a few people reported oral sex practices. As for the anorectum, only MSM were tested and more than 20% of the rectal samples tested positive for *C. trachomatis* (6/28). According to a study conducted by Peters, R. P. H. et al, 2011 [176] in STD clinic visitors, almost all cases of oropharyngeal and anorectal infection were asymptomatic and would thus have been missed if screening was only performed on the basis of reported symptoms. Also, in the same study, one-third of infected women had the oropharynx as the only site of infection and the clinical relevance of this observation was directly related to the risk of transmission from oropharynx to penis with fellatio. Therefore, there's an essential necessity to adjust screening practices of anatomical sites based on sexual history rather than over a symptom-based approach.

Through our study we were able to find an association between *C. trachomatis* infection and sexual orientation (p=0.002), which we suggest that may be in accordance with European reports that describe that 88% of the cases are due to heterosexual transmission [66].

Coinfections of *C. trachomatis* with *N. gonorrhoeae* and *M. genitalium* are to be expected [78, 120] due to similar situations of risk of acquisition – where sexual behavior plays a major role [2, 7, 105].

N. gonorrhoeae was the second most frequent STI, with a frequency of 6.6%, and its increasing numbers according to the ECDC and CDC [13, 96] may also be related to AMR, as strains exhibiting multidrug resistance (MDR) and extensive drug resistance (XDR) are expected [94]. Gonococcal infection of the rectum and oropharynx can occur in both sexes depending on the sexual practices, but is predominantly found among MSM [94]. However, since oral and anal intercourse have been increasing in the latest years among heterosexual couples [10] and more 'experimental' sex behavior and practices are being noticed, adding several possible transmission routes, *N. gonorrhoeae* infection prevalence rates are expected to rise. Having multiple sexual partners and lack of consistent condom use even during oral sex practices, among others, also contribute for rising rates of *N. gonorrhoeae*.

N. gonorrhoeae is usually three times more common in men than in women [66]. In our study, we were able to observe that men contributed with more than two thirds of the infected and 53.2 % of them were MSM; in fact, a statistically significant association between sexual orientation and current gonococcal infection (p=0.002) could be established [2, 9, 94].

N. gonorrhoeae infection was associated with age (p<<0.001), with the age range of 20 to 24 years old accounting for more than one third of the detected gonococcal infections (24/68, 35.3%); this age range is usually related with the highest prevalence of *N. gonorrhoeae* [7]. Also, the ECDC [66] accounts individuals with ages between 15 to 24 years as contributing for 39% of the reported cases; our findings are in concordance with those estimations.

Considering the motives that led people to the STI clinic, we observed a statistically significant association with *N. gonorrhoeae* infection (p<<0.001); thus, although this infection may cause asymptomatic cases [98], at least some kind of discomfort seem to lead people infected with *N. gonorrhoeae* to healthcare providers. In fact, in comparison to *C. trachomatis,* gonococcal infections tend to cause a stronger inflammatory response and, in men, the majority of urethral infections origin urethritis with painful urination [13], which may suggest an association between the existence of symptoms and being infected with *N. gonorrhoeae*.

M. genitalium frequency, on the other hand, was of 2.6%, a low positivity that has been described when comparing to other studies with the same-type of population [184, 185, 186]. One possible reason for this result would be the indiscriminate use of azithromycin for other infections (e.g. respiratory) that would treat *M. genitalium* in an accessory fashion; however, as the same impact doesn't seem to prevail for *C. trachomatis*, the reason is surely different, namely, specificities of the Portuguese *M. genitalium* epidemiological scenario, which remains unknown.

M. genitalium is more commonly found in women rather than men and of younger ages [119]; however, in this study, less than one third of the infected patients were women (8/27), and most of the infected patients are older than 25 years old (18/27, 66.7%) and, again, this might be due to the nature of our study population. In fact, for the few detected infections, no statistically significant association could be established with any other variable under evaluation.

T. vaginalis frequency was low (0.5%), which could be expected, considering that it is much more frequent in women due to their biological specificities [145], and less than one third of our study population were females; however, 80% of the detected cases were in women, and among women *T. vaginalis* represented 1.4% (4/294). Although the incidence of *T. vaginalis* infection may vary according to many factors (including age, sexual activity, number of sexual partners, presence of other STIs, among others) [135], a previously published study has reported that *T. vaginalis* prevalence may be as high as 26.2% for symptomatic women and 6.5% among asymptomatic women screened [187]. According to other smaller studies, its prevalence was estimated between 13.0% and 15.0% among women attending STD clinics [188, 189]. There are large differences in the prevalence of *T. vaginalis* infections between different regions, with the highest prevalence described in Africa (20.2% of females, 2% of males) and the lowest prevalence found in Europe (5.8% of females, 0.6% of males) [134]. In our study, a very low prevalence was also found but, since there aren't any other studies regarding this matter in Portugal, we can't identify the underlying reasons that explain the low percentage of cases in our study population. However, it can be suggested that the usage of antiseptics and/or the intake of oral contraceptives (they reduce the vaginal pH variation in the host) may contribute to reduce the number of *T. vaginalis* infections, therefore justifying the low rates observed.

As usually described [146], most of the cases occurred in older patients, and all in urethral samples which contain enough vaginal secretion for detecting the protozoan by NAATs [158, 160].

It is known that STIs and their repeated episodes may cause epithelial erosions and alter host immune defenses, thereby increasing susceptibility to acquire and transmit HIV infections [105]; yet, in this study and despite that about 7.0% of the studied population was infected with HIV, no statistically significant association between any current STI studied and HIV positivity was found; however, it is of note that 'never-tested patients' for HIV were included in the HIV-negative category and this approach might have influenced the analysis. According to this hypothesis, among all the HIV-positive people, 45 (67.2%) had at least one STI in the past, in contrast with HIV-negative people, for which only 229 (24.9%) had a STI in the past. This suggests that these past STIs may have increased the susceptibility to acquire HIV. It is possible that these infections are not only causally related to HIV, but they may also be confounders in the relationship between behavioral risks and HIV acquisition [105]. Nonetheless, 67 is still a very high number of HIV positives, considering the national prevalence is 9.6 cases per 100.000 inhabitants [190].

Considering that STIs control relies on appropriate screening and treatment, paired with targeted prevention efforts, use of effective diagnostics, partner notification and epidemiological surveillance [94], this study constituted a contribution to the knowledge of major curable STIs in attendees of a STI clinic. Early diagnose and appropriate therapy provided to those patients, will potentially reduce long-term complications caused by STIs.

2. Evaluation of the distribution of *C. trachomatis ompA*-genotypes among the collection of the Portuguese national institute of health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, IP)

2.1. Characterization of *C. trachomatis* clinical strains

Since the year of 1990 to the present year, 2579 *C. trachomatis ompA*-positive samples were included in the INSA's national database. Most of those samples are either from the endocervix and from the urethra (including urines), followed by anorectal, oropharyngeal and, conjunctiva with only few samples.

C. trachomatis ompA-positive samples were originated from the routine diagnosis at INSA and other laboratories that gently sent their samples to INSA. Thus, the majority were from adults, at anogenital sites, and more recently (year of 2015), oropharynx samples also began to diversify the collection. Ocular samples, although could have been obtained from adults, were mostly obtained from newborns whose mothers were infected with *C. trachomatis*, and got infected by vertical transmission, from the mother to the child during passage through the infected birth canal; the rate of transmission has been established in 50 to 70% [191].

2.2. Genotypes analysis

At INSA, over the years, different methodologies were used for typing, but they were always based in *ompA* gene amplification. In the 1990s, amplicons were analyzed by RFLP [192], but this time-consuming technology was replaced by sequencing as soon as Sanger sequencing became available at INSA. This changing doesn't impact over results as all strains previously typed with RFLP were re-typed by sequencing and this result prevailed, as RFLP didn't discriminate many of the *ompA*-genotypes. The 368 strains that failed to be typed using Sanger sequencing (samples and/or DNA lost or degraded over time) were excluded from further analysis.

In general, the frequency of *C. trachomatis ompA*-genotypes oscillates along the years; however, it has been relatively constant in the host lab (**Figure 3.13**) [41, 72, 192] and in other countries [193, 194, 195].

OmpA-genotype B has been essentially found in trachoma-endemic communities of developing countries, ranging from Latin America to Sub-Saharan Africa or Asia [196]. We could speculate that its prevalence in our population could be due to travelers who got infected in those areas. However, it is hard to explain why the same phenomenon doesn't occur with A and C, *ompA*-genotypes also related to trachoma [2, 33, 197].

OmpA-genotypes D to K are most often associated with chlamydial infections acquired from vaginal intercourse and their distribution by age groups revealed relatively similar. Genotype E, was the most common genotype for both men and women, as predicted and referred by previous studies [48, 192]. Together with genotype F, the second most common *ompA*-genotype, are the most frequent in this Portuguese collection, representing 50% of all *C. trachomatis* strains, and they are considered as the most successful *C. trachomatis ompA*-genotypes [41]. The reasons for this remarkable success remain unclear, although it has been speculated that their advantage may reside in a more effective host immune invasion and/or transmission capacity [198]. The genomic features underlying their infection capacity involve several genomic regions as a separate branch is usually obtained [199]; that is not the case for the *ompA* in which they differ by 15% but phylogenetically are put in different branches [33, 199].

OmpA-genotypes D and E were the only ones represented in every site of infection. Despite the high prevalence of F, it was not detected in the oropharynx; this may be explained by the low number of specimens from that anatomical site. *ompA*-genotype G, while less frequent, was detected in the oropharynx, where a statistical association could be speculated to exist, according to a previous study [10], with being MSM – along with genotypes D and J in MSM. *OmpA*-genotype G became the fourth most common genotype since the beginning of 2000, although the reasons underlying this finding are still to be fully understood [10, 41].

LGV was endemic in Africa, Southeast Asia, Central and South America and in the Caribbean, while rarely reported in developed countries [200]. At INSA, *ompA*-genotype L3 was never found and L1 was detected once. Then, 13 years ago, an LGV outbreak occurred among MSM in The Netherlands, and although in that same year, nothing of a kind could be detected in Portugal, L2 strains have been identified since 2007 becoming quite frequent from the year 2015. This may be due to the high number of MSM attending to STI clinics collaborating with INSA, which increased *C. trachomatis* testing in anorectal samples where L2 is the most frequent genotype. Together with the other three genotypes related to MSM – D, G and J – they are more frequent in men than in women, suggesting a putative tissue tropism of those strains for the rectum epithelia [10]. *ompA*-genotype L2 was more common among people of 25 years and older, a finding most probably related with the age range of MSM attending to STI clinics in Lisbon.

ompA-genotype H, on the opposite, is more frequent in women than in men and, as described by Borrego M. J., et al., 1997[192] and it was highly frequent by the end of the 1990s, when it could be related to a sexual network involving prostitutes and their clients; since that prostitution area changed, about 15 years ago, *ompA*-genotype H frequency decreased [41].

Mixed infections, for which the exact *ompA*-genotype could not be identified, could not be associated with any particular situation. According to Barnes, R. C. et al., 1987 [201], it is estimated that approximately 2 – 5% of chlamydial genital infections occur as mixed infections, possibly due to different episodes of infection and the lack of cross protection between genotypes [202]. This can also lead to genetic recombination, occurring readily, which may impact disease severity [203].

2.3. Sub-types and variants analysis

The appearance of different genetic variants might be due to tissue tropism, whereby *C. trachomatis ompA*-genotypes propagate better in urogenital tissues or in anorectal tract epithelia, due to genetic exchanges [204] or simply due to point mutations that might induce a better fitness to avoid host protection schemes [41, 203]. Molecular epidemiology studies are important contributions for evaluating changes in *ompA*-genotype frequency that might be related with specific clinical features and/or population itself that may influence the occurrence of variants. However, the exact reasons behind variation are hard to pinpoint.

OmpA-sequencing, enables to detect not only the genotype of each strain, but also strain-variants. A variant corresponds to a nucleotide changing in relation to the described for the same position in the sequence of the prototype strain of a given *ompA*-genotype [41].

It is important to note that prototype strains (also designed as reference strains) did not get this status because they are the most common circulating type; they were simply the first strain described for each *C. trachomatis ompA*-genotype, and became 'popular' because they could be used in several research studies, in different laboratories, and applications and results would be comparable. So, it is possible for a reference strain to be, itself, quite rare, or no longer circulating. Accordingly, from the 824/2211 (37.3%) clinical specimens of INSA's collection many variants were found, and C and I prototype strains were never detected. We could speculate that rare and less common variants may not have found the best fitting for their MOMP, and for that reason keep undergoing adaptation processes. In contrast, the most successful genotypes, E and F, among which the number of variants is much lower; in fact, several studies [33, 205, 206] report a high level of conservation among E and F strains. A better fitness of these genotypes would favor some sort of less forceful host immune response, enabling microorganisms to go undetected (or less detected), leading to further dissemination and contributing for high epidemiological rates. Moreover, as a consequence of any mutation, the microorganism must undergo adaptations that are energy consuming, and the maintenance of any mutation only succeeds when it beneficiates the microorganism, allowing it to escape from defense mechanisms of the host; this could be a reason for some mutations to persist while others are soon eliminated from the microbial population [41].

In the present study, 123 distinct types of genetic variants were detected. Among these, 31 mutations (25.2%) were synonymous (i.e. codon changes but codes for the same amino acid), 87.1% (27/31) of these synonymous mutations occurred in conserved domains; the remaining 92 were non-synonymous, meaning that the new codon will imply a different amino acid. These phenomena reflect the plasticity of MOMP, and could have an impact at whole protein level, on the conformation of the protein [41]. In the present study, 37.0% (34/92) of these non-synonymous mutations occurred in a conserved domain, while the majority, 63.0% (58/92), occurred in variable domains, again putatively inducing changes in specific epitopes and contributing to evasion from host immune defense mechanisms. The most common regions where mutations were observed were VDIV and VDII, followed by CDIII. This may be explained in regard to the structure of the MOMP itself, since it is not clear whether other residues, in addition to the located in the four VDs, are externally exposed when the MOMP conformation is intact. Thus, although the MOMP porin structure incorporates a canonical 16-stranded form, it may have novel oligomeric or dynamic structural changes accounting for the discrepancies observed [207].

No relation could be established between the anatomical site of infection and variability since the number of analyzed samples was not even among regions. However, we estimated that variant strains occur natural and randomly in nature, according to what was described by Nunes, A. et al., 2009 [41]. In fact, *C. trachomatis* has been going through processes of co-evolution with humans for many years, and the phenomenon of emerging mutations end-up fixating on the population likely occur as a biological advantage in terms of infectivity and transmission, or they would get lost within time, according to the biological adaptive needs of the bacteria.

3. Evaluation of intra-patient *C. trachomatis* genetic heterogeneity affecting homopolymeric tracts potentially driving phase variation, among *C. trachomatis*-positive DNA samples selected from the collection of the Portuguese national institute of health (Instituto Nacional de Saúde Doutor Ricardo Jorge, INSA, IP)

Advances in NGS-based methodologies and associated bioinformatics now enable an in-depth investigation of the genetic heterogeneity generated by pathogens, on the course of the human infection. In this field, focus have been done on unveiling intra-host phase variation-driven phenotypic changes, usually mediated by changes in the length of homopolymeric tracts (poly (Ns)), which is a well-known mechanism used by pathogenic bacteria to promote tissue adaptation or virulence. In this study, we aimed to scale-up the study of the genetic heterogeneity affecting poly (Ns) of the *C. trachomatis* genome in the context of human infection, being particularly interested in unveiling potential mechanisms of phase variation in *C. trachomatis*. To achieve this, we applied a strategy focused on deep sequencing (directly from clinical samples) of amplicon products (amplicons) targeting specific homopolymeric tracts. Our results markedly reinforce the current knowledge on the both inter and intra-patient genetic heterogeneity affecting poly(Ns), potentially driving phase variation within human *in vivo C. trachomatis* populations, which could constitute novel research lines towards the understanding of this bacteria's prevalence, tissue tropism or clinical outcomes.

As a remarkable example, the cytotoxin gene CT166, which is one gene previously pointed out as a potential target of phase variation, revealed, in the present study, an unequivocal intra-strain variability. This finding strongly consolidates the hypothesis that reversible poly (N)- driven genetic changes in this gene may underlie the regulation of the *in vivo* cytotoxin functionality through an ON/OFF mechanism of phase variation. While it is well-known [60, 166] that CT166 displays a dissimilar presence/absence profile among *C. trachomatis* strains [51] (it is absent in all LGV genotypes but present in genital *ompA*-genotypes), our study reinforces that *C. trachomatis* may be able to activate/inactivate this virulence factor on the course of the human infection. This is particularly relevant as CT166/cytotoxin is believed to act on the rapid disassembly of cytoskeleton actin filaments during bacterial internalization process [209, 210], and it has been hypothesized its association not only with virulence, but also with *C. trachomatis* tissue tropism. Although we were not able to infer the latter hypothesis, since the number of strains belonging to non-genital anatomical sites were scarce, our results open new clues suggesting that the variable poly (N) may be a key for this role [60].

Although phase variation mediated by variable homopolymeric tracts in *C. trachomatis* is a subject to be further studied, we investigated other potential targets regarding their inter- and intra-patient heterogeneity, and found distinct trends: **a)** Low intra-patient variability with clade-specific dominant profile – suggesting that they constitute genes that are not subjected to phase variation. Instead, the differential poly (N) profile might have been a result of ancestral deletions/insertions that have been vertically inherited, being currently shared by all the same-clade isolates [60]. An example of this are the homopolymeric tracts affecting CT823 gene that encodes for a serine protease, which interacts with the host cell by manipulating the signal pathways [211]. **b)** Low intra-strain variability without clade-specific dominant profile, i.e., they constitute conserved homopolymeric tracts with little evidence of being drivers of intra-patient *C. trachomatis* phase variation, although they show non-negligible inter-strain diversity. An example of this tendency are the homopolymeric tracts affecting CT694, which codes for a protein that acts as substrate for *C. trachomatis* type 3 secretion system and is an early cycle-associated potential virulence factor [212]. **c)** High intra-strain variability, without clade-specific dominant profile – suggesting the existence of phase variation mediated by variable homopolymeric tracts. These can be pointed out as good targets for more studies in this area. An example of this are the homopolymeric tracts affecting CT259 that codes a protein phosphatase associated with functional kinase-based signaling cascade for interaction with host signaling pathways [213]. Remarkably, the poly (G/C) tracts likely affecting the CT259 putative regulatory region was the most variable target from our selection, especially in strains belonging to clade T1.

Of note, no relation could be established between gender and dominant profile, neither a relation between anatomical site of infection and dominant profile, but in this case, it is mostly due to the low number of strains from other regions besides the genital site of infection.

In summary, although this study constitutes an unequivocal turning point on our knowledge of the intra- and inter-patient heterogeneity of poly (G) (potentially regulating the ON/OFF state of *C. trachomatis* proteins in the context of human infection), a future scale-up of this approach to an even and larger collection of *C. trachomatis*-positive DNA samples, as well as to other genes, will be pivotal to consolidate some of the hypothesis raised in this chapter of the master thesis.

Final Remarks and Future Perspectives

Almost a fifth of the population involved in the first study was diagnosed with, at least, one STI (*C. trachomatis, N. gonorrhoeae, M. genitalium* or *T. vaginalis*), among which, the most frequent was *C. trachomatis*. Considering that this study only involved patients from one STI clinic, it highlights the need for a similar evaluation nationwide as, to date, there are no information concerning the prevalence of these STIs. This knowledge can be considered fundamental for the implementation of specific prevention measures.

C. trachomatis was selected for further characterization, concerning the *ompA*-genotypes detected in the host laboratory since the early 1990's. As predicted by previous worldwide studies, *ompA*-genotype E, was the most common for both men and women, followed by genotype F. Both are considered as the most successful *C. trachomatis ompA*-genotypes for which the number of *ompA*-variants is much lower when compared to other *ompA*-genotypes. A better fitness of E and F would favor them to go somehow undetected by the host defense mechanisms, facilitating dissemination and consequently leading to higher epidemiological rates. Indeed, there was a statistically significant association between *ompA*-genotypes and the anatomical site of infection ($p<<0.001$), also suggesting that *ompA*-genotype L2 could be associated with the anorectal infection and genotype G with the oropharynx. However, genomic studies involving the whole genome sequence together with clinical data are required for a more complete understanding of the genetic features of circulating *C. trachomatis* strains.

Regarding phase-variation mechanisms, the cytotoxin gene CT166 revealed to be an unequivocal target of intra-strain variability. In addition, we could show, for the first time, that there are other high intra-strain variability homopolymeric tracts, such as the one affecting CT259, suggesting the existence of phase variation phenomena in *C. trachomatis*. A future scale-up of this approach, using an homogeneous and wider range of *C. trachomatis*-positive DNA samples together with their clinical background, along with putative phase variation related genes, should be performed in order to consolidate some of the hypothesis raised through the present master thesis.

References

[1] Newman, L., Rowley, J., Hoorn, S. Vander, Wijesooriya, N. S., Unemo, M., et. al (2015). Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. *PLoS ONE*, *10*(12), 1–17. http://doi.org/10.1371/journal.pone.0143304

[2] Unemo, M., Ballard, R., Ison, C., Lewis, D., Ndowa, F., & Peeling, R. (2013). Laboratory diagnosis of sexually transmitted infections, including human immunodeficiency virus. *World Health Organization*.

[3] Casillas-vega, N., Morfín-otero, R., García, S., Llaca-díaz, J., Mendoza-olazarán, S., Flores-treviño, S., & Petersen-morfín, S. (2016). Sexually transmitted pathogens, coinfections and risk factors in patients attending obstetrics and gynecology clinics in Jalisco, Mexico. *Salud Pública de México*, *58*(4), 437–445.

[4] Workowski, K. A., & Bolan, G. A. (2015). Sexually Transmitted Diseases Treatment Guidelines, 2015. *Morbidity and Mortality Weekly Report - CDC*, *64*(3).

[5] Global health sector strategy on sexually transmitted infections 2016-2021. (2016). *World Health Organization*.

[6] Garbers, S., Friedman, A., Martinez, O., Scheinmann, R., Bermudez, D., Silva, M., et. al (2016). Adapting the Get Yourself Tested Campaign to Reach Black and Latino Sexual-Minority Youth. *Health Promotion Practice*, *17*(5), 1–12. http://doi.org/10.1177/1524839916647329

[7] Flagg, E., Kidd, S., Presley, R., & Stenger, M. (2015). Sexually Transmitted Disease Surveillance 2014. *Centers for Disease Control and Prevention*.

[8] Muzny, C. A., Sunesara, I. R., Martin, D. H., & Mena, L. A. (2011). Sexually Transmitted Infections and Risk Behaviors Among African American Women Who Have Sex With Women: Does Sex With Men Make a Difference? *Sexually Transmitted Diseases*, *38*(12), 1118–1125. http://doi.org/10.1097/OLQ.0b013e31822e6179

[9] Workowski, K. A., & Berman, S. M. (2011). Centers for disease control and prevention sexually transmitted disease treatment guidelines. *Clinical Infectious Diseases*, *53*(S3), 59–63. http://doi.org/10.1093/cid/cir694

[10] Christerson, L., Bom, R. J. M., Bruisten, S. M., Yass, R., Hardick, J., Bratt, G., et. Al (2012). *Chlamydia trachomatis* Strains Show Specific Clustering for Men Who Have Sex with Men Compared to Heterosexual Populations in Sweden, the Netherlands, and the United States Linus. *Journal of Clinical Microbiology*, *50*(11), 3548–3555. http://doi.org/10.1128/JCM.01713-12

[11] Stevens, M. P., Twin, J., Fairley, C. K., Donovan, B., Tan, S. E., Yu, J., et. al (2010). Development and Evaluation of an *ompA* Quantitative Real-Time PCR Assay for *Chlamydia trachomatis* Serovar Determination. *Journal of Clinical Microbiology*, *48*(6), 2060–2065. http://doi.org/10.1128/JCM.02308-09

[12] Ness, R. B., Soper, D. E., Richter, H. E., Randall, H., Peipert, J. F., Nelson, D. B., et. al (2008). Chlamydia Antibodies, Chlamydia Heat Shock Protein, and Adverse Sequelae After Pelvic

Inflammatory Disease: The PID Evaluation and Clinical Health (PEACH) Study. *Sexually Transmitted Diseases*, *35*(2), 129–135. http://doi.org/10.1097/OLQ.0b013e3181557c25

[13] Papp, J. R., Schachter, J., Gaydos, C. A., & Pol, B. Van Der. (2012). Recommendations for the Laboratory-Based Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* — 2014 John. *Morbidity and Mortality Weekly Report - CDC*, *100*(2), 130–134. http://doi.org/10.1016/j.pestbp.2011.02.012.Investigations

[14] Nunes, A., & Gomes, J. P. (2014). Evolution, phylogeny, and molecular epidemiology of Chlamydia. *Infection, Genetics and Evolution*, *23*, 49–64. http://doi.org/10.1016/j.meegid.2014.01.029

[15] Knittler, M. R., Radomski, N., Einenkel, R., & Anne, M. (2016). Chlamydia – host cell interaction not only from a bird's eye view: some lessons from *Chlamydia psittaci*. *FEBS Letters*, *590*, 3920–3940. http://doi.org/10.1002/1873-3468.12295

[16] Abdelrahman, Y. M., & Belland, R. J. (2005). The chlamydial developmental cycle. *FEMS Microbiology Reviews*, *29*, 949–959. http://doi.org/10.1016/j.femsre.2005.03.002

[17] Darville, T. (2005). *Chlamydia trachomatis* Infections in Neonates and Young Children. *Seminars in Pediatric Infectious Diseases*, *16*(4), 235–44. http://doi.org/10.1053/j.spid.2005.06.004

[18] Nans, A., Ford, C., & Hayward, R. D. (2015). Host-pathogen reorganisation during host cell entry by *Chlamydia trachomatis*. *Microbes and Infection*, *17*(11–12), 727–731. http://doi.org/10.1016/j.micinf.2015.08.004

[19] Carabeo, R. (2011). Bacterial Subversion of Host Actin Dynamics at the Plasma Membrane. *Cell Microbiology*, *13*(10), 1460–1469. http://doi.org/10.1111/j.1462-5822.2011.01651.x.Bacterial

[20] Elwell, C., Mirrashidi, K., & Engel, J. (2016). Chlamydia cell biology and pathogenesis. *Nature Reviews Microbiology*, *14*(6), 385–400. http://doi.org/10.1038/nrmicro.2016.30

[21] Abdelrahman, Y., Ouellette, S. P., Belland, R. J., & Cox, J. V. (2016). Polarized Cell Division of *Chlamydia trachomatis*. *PLoS Pathogens*, *12*(8), 1–20. http://doi.org/10.1371/journal.ppat.1005822

[22] Zuck, M., Ellis, T., Venida, A., & Hybiske, K. (2017). Extrusions are phagocytosed and promote Chlamydia survival within macrophages. *Cellular Microbiology*, *19*(October 2016), 1–12. http://doi.org/10.1111/cmi.12683

[23] Hybiske, K., & Stephens, R. S. (2007). Mechanisms of host cell exit by the intracellular bacterium *Chlamydia*. *Proceedings of the National Academy of Sciences*, *104*(27), 11430–11435. http://doi.org/10.1073?pnas.0703218104

[24] Ziklo, N., Huston, W. M., Hocking, J. S., & Timms, P. (2016). *Chlamydia trachomatis* Genital Tract Infections: When Host Immune Response and the Microbiome Collide. *Trends in Microbiology*, *24*(9), 750–765. http://doi.org/10.1016/j.tim.2016.05.007

[25] Hogan, R. J., Mathews, S. A., Mukhopadhyay, S., Summersgill, J. T., & Timms P. (2004). Chlamydial persistence: beyond the biphasic paradigm. *Infection and Immunity, 72*(4), 1843–1855.

[26] Aistleitner, K., Anrather, D., Schott, T., Klose, J., Bright, M., Ammerer, G., & Horn, M. (2015). Conserved features and major differences in the outer membrane protein composition of chlamydiae. *Environmental Microbiology*, *17*(4), 1397–1413. http://doi.org/10.1111/1462-2920.12621

[27] Caldwell, H. D., Kromhout, J., & Schachter, J. (1981). Purification and Partial Characterization of the Major Outer Membrane Protein of *Chlamydia trachomatis*. *Infection and Immunity*, *31*(3), 1161–1176.

[28] Nikaido, H. (2003). Molecular Basis of Bacterial Outer Membrane Permeability Revisited. *Microbiology and Molecular Biology Reviews*, *67*(4), 593–656. http://doi.org/10.1128/MMBR.67.4.593

[29] Liu, X., Afrane, M., Clemmer, D. E., Zhong, G., & Nelson, D. E. (2010). Identification of *Chlamydia trachomatis* Outer Membrane Complex Proteins by Differential Proteomics. *Journal of Bacteriology*, *192*(11), 2852–2860. http://doi.org/10.1128/JB.01628-09

[30] Baehr, W., Zhang, Y., Joseph, T., Su, H., Nano, F. E., Everettt, K. D. E., et. al (1988). Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. *Proceedings of the National Academy of Sciences*, *85*(June), 4000–4004.

[31] Yuan, Y., Zhang, Y., Watkins, N. G., & Caldwell, H. D. (1989). Nucleotide and Deduced Amino Acid Sequences for the Four Variable Domains of the Major Outer Membrane Proteins of the 15 *Chlamydia trachomatis* Serovars. *Infection and Immunity*, *57*(4), 1040–1049.

[32] Khattab, R. A., & Abdelfattah, M. M. (2016). Study of the prevalence and association of ocular chlamydial conjunctivitis in women with genital infection by *Chlamydia trachomatis*, *Mycoplasma genitalium* and *Candida albicans* attending outpatient clinic. *International Journal of Ophthalmology*, *9*(8), 1176–1186. http://doi.org/10.18240/ijo.2016.08.15

[33] Lysén, M., Osterlund, A., Rubin, C., Persson, T., Persson, I., & Anders, O. (2004). Characterization of ompA Genotypes by Sequence Analysis of DNA from All Detected Cases of *Chlamydia trachomatis* Infections during 1 Year of Contact Tracing in a Swedish County. *Journal of Clinical Microbiology*, *42*(4), 1641–1647. http://doi.org/10.1128/JCM.42.4.1641

[34] Morré, S. A., Rozendaal, L., van Valkengoed, I. G. M., Boeke, A. J. P., van Voorst Vader, P. C., Schirm, J., et. al (2000). Urogenital *Chlamydia trachomatis* Serovars in Men and Women with a Symptomatic or Asymptomatic Infection: an Association with Clinical Manifestations? *Journal of Clinical Microbiology*, *38*(6), 2292–2296.

[35] Bom, R. J. M., Christerson, L., Schim Van Der Loeff, M. F., Coutinho, R. A., Herrmann, B., & Bruisten, S. M. (2011). Evaluation of high-resolution typing methods for *Chlamydia trachomatis* in samples from heterosexual couples. *Journal of Clinical Microbiology*, *49*(8), 2844–2853. http://doi.org/10.1128/JCM.00128-11

[36] Bauwens, J. E., Lampe, M. F., Suchland, R. J., Wong, K., & Stamm, W. E. (1995). Infection with *Chlamydia trachomatis* lymphogranuloma venereum serovar L1 in homosexual men with proctitis: molecular analysis of an unusual case cluster. *Clinical Infectious Diseases, 20*, 576 –581.

[37] Pedersen, L. N., Herrmann, B., & Møller, J. K. (2009). Typing *Chlamydia trachomatis*: from egg yolk to nanotechnology. *FEMS Immunology and Medical Microbiology*, *55*(1), 120–130. http://doi.org/10.1111/j.1574-695X.2008.00526.x

[38] Currie, M. J., & Bowden, F. J. (2007). The importance of chlamydial infections in obstetrics and gynaecology: An update. *Australian and New Zealand Journal of Obstetrics and Gynaecology*, *47*(1), 2–8. http://doi.org/10.1111/j.1479-828X.2006.00670.x

[39] Caldwell, H. D., Wood, H., Crane, D., Bailey, R., Jones, R. B., Mabey, D., et. al (2003). Polymorphisms in *Chlamydia trachomatis* tryptophan synthase genes differentiate between genital and ocular isolates. *Journal of Clinical Investigation*, *111*(11), 1757–1769. http://doi.org/10.1172/JCI200317993

[40] Nunes, A., Borrego, M. J., & Gomes, J. P. (2013). Genomic features beyond *Chlamydia trachomatis* phenotypes: What do we think we know? *Infection, Genetics and Evolution*, *16*, 392–400. http://doi.org/10.1016/j.meegid.2013.03.018

[41] Nunes, A., Borrego, M. J., Nunes, B., & Florindo, C. (2009). Evolutionary Dynamics of *ompA*, the Gene Encoding the *Chlamydia trachomatis* Key Antigen. *Journal of Bacteriology*, *191*(23), 7182–7192. http://doi.org/10.1128/JB.00895-09

[42] Quint, K., Porras, C., Safaeian, M., Gonza, P., Hildesheim, A., Quint, W., et. al (2007). Evaluation of a Novel PCR-Based Assay for Detection and Identification of *Chlamydia trachomatis* Serovars in Cervical Specimens. *Journal of Clinical Microbiology*, *45*(12), 3986–3991. http://doi.org/10.1128/JCM.01155-07

[43] Andersson, J. O., & Andersson, S. G. E. (1999). Insights into the evolutionary process of genome degradation. *Current Opinion in Genetics & Development*, *9*(6), 664–671.

[44] Stephens, R. S., Kalman, S., Lammel, C., Fan, J., Marathe, R., Aravind, L., et. al (1998). Genome Sequence of an Obligate Intracellular Pathogen of Humans: *Chlamydia trachomatis*. *Science*, *282*(5389), 754–759.

[45] Gomes, P., Nunes, A., Bruno, W. J., Borrego, M. J., Florindo, C., & Dean, D. (2006). Polymorphisms in the Nine Polymorphic Membrane Proteins of *Chlamydia trachomatis* across All Serovars: Evidence for Serovar Da Recombination and Correlation with Tissue Tropism. *Journal of Bacteriology*, *188*(1), 275–286. http://doi.org/10.1128/JB.188.1.275

[46] Hentschel, U., & Steinert, M. (2001). Symbiosis and pathogenesis: common themes, different outcomes. *Trends in Microbiology*, *9*(12), 2260.

[47] Clarke, I. N. (2011). Evolution of *Chlamydia trachomatis*. *Annals of the New York Academy of Sciences*, *1230*, 11–18. http://doi.org/10.1111/j.1749-6632.2011.06194.x

[48] Hadfield, J., Harris, S. R., Seth-smith, H. M. B., Parmar, S., Andersson, P., Giffard, P. M., et. al (2017). Comprehensive global genome dynamics of *Chlamydia trachomatis* show ancient diversification followed by contemporary mixing and recent lineage expansion. *Genome Research*, *27*(7), 1–10. http://doi.org/10.1101/gr.212647.116

[49] Read, T. D., Brunham, R. C., Shen, C., Gill, S. R., Heidelberg, J. F., White, O., et. al (2000). Genome sequences of *Chlamydia trachomatis* MoPn and *Chlamydia pneumoniae* AR39. *Nucleic Acids Research*, *28*(6), 1397–1406.

[50] Taylor, L. D., Nelson, D. E., Dorward, D. W., Whitmire, W. M., & Caldwell, H. D. (2010). Biological Characterization of *Chlamydia trachomatis* Plasticity Zone MACPF Domain Family Protein CT153. *Infection and Immunity*, *78*(6), 2691–2699. http://doi.org/10.1128/IAI.01455-09

[51] Carlson, J. H., Hughes, S., Hogan, D., Cieplak, G., Sturdevant, D. E., Mcclarty, G., et. al (2004). Polymorphisms in the *Chlamydia trachomatis* Cytotoxin Locus Associated with Ocular and Genital Isolates. *Infection and Immunity*, *72*(12), 7063–7072. http://doi.org/10.1128/IAI.72.12.7063

[52] Byrne, G. I. (2015). *Chlamydia trachomatis* strains and virulence: rethinking links to infection prevalence and disease severity. *Journal of Infectious Diseases*, *201*, S126–S133. http://doi.org/10.1086/652398.

[53] Belland, R. J., Scidmore, M. A., Crane, D. D., Hogan, D. M., Whitmire, W., Mcclarty, G., & Caldwell, H. D. (2001). *Chlamydia trachomatis* cytotoxicity associated with complete and partial cytotoxin genes. *Proceedings of the National Academy of Sciences*, *98*(24), 13984–13989.

[54] Jewett, T. J., Fischer, E. R., Mead, D. J., & Hackstadt, T. (2006). Chlamydial TARP is a bacterial nucleator of actin. *Proceedings of the National Academy of Sciences*, *103*(42), 15599–15604.

[55] Valdivia, R.H. (2008). Chlamydia effector proteins and new insights into chlamydial cellular microbiology. *Current Opinion in Microbiology, 11*, 53–59

[56] Dai, W., & Li, Z. (2014). Conserved type III secretion system exerts important roles in *Chlamydia trachomatis*. *International Journal of Clinical and Experimental Pathology*, *7*(9), 5404–5414.

[57] Hueck, C. J. (1998). Type III Protein Secretion Systems in Bacterial Pathogens of Animals and Plants. *Microbiology and Molecular Biology Reviews*, *62*(2), 379–433.

[58] Henderson, I. R., & Lam, A. C. (2001). Polymorphic proteins of *Chlamydia* spp. – autotransporters beyond the Proteobacteria. *Trends in Microbiology*, *9*(12), 573–578.

[59] Nguyen, B. D., Cunningham, D., Liang, X., Chen, X., Toone, E. J., Raetz, C. R. H., & Zhou, P. (2011). Lipooligosaccharide is required for the generation of infectious elementary bodies in *Chlamydia trachomatis*. *Proceedings of the National Academy of Sciences*, *108*(25), 10284–10289. http://doi.org/10.1073/pnas.1107478108

[60] Borges, V., Pinheiro, M., Antelo, M., Sampaio, D. A., & Vieira, L. (2015). *Chlamydia trachomatis* In Vivo to In Vitro Transition Reveals Mechanisms of Phase Variation and Down-Regulation of Virulence Factors. *PLoS ONE*, *10*(7), 1–31. http://doi.org/10.1371/journal.pone.0133420

[61] Nunes, A., Gomes, P., Karunakaran, K. P., & Brunham, R. C. (2015). Bioinformatic Analysis of *Chlamydia trachomatis* Polymorphic Membrane Proteins PmpE, PmpF, PmpG and PmpH as Potential Vaccine Antigens. *PLoS ONE*, *10*(7), 1–16. http://doi.org/10.1371/journal.pone.0131695

[62] van Belkum, A., Scherer, S., van Alphen, L., & Verbrugh, H. (1998). Short-Sequence DNA Repeats in Prokaryotic Genomes. *Microbiology and Molecular Biology Reviews*, *62*(2), 275–293.

[63] Levinson, G. & Gutman, G.A. (1987). Slipped-Strand Mispairing: A Major Mechanism for DNA Sequence Evolution. *Molecular Biology and Evolution, 4*(3), 203-221.

[64] Salaün, L., Snyder, L. A., & Saunders, N. J. (2003). Adaptation by phase variation in pathogenic bacteria. *Advances in Applied Microbiology, 52,* 263-301.

[65] Stamm, W. E. *Harrison's Principles of Internal Medicine*, 15th edition. Columbus, OH: McGraw-Hill, 2001.

[66] Amato-Gauci, A. J., & Spiteri, G. (2015). Sexually transmitted infections in - 2013. http://doi.org/10.2900/910982

[67] Davies, B., & Ward, H. (2016). Guidance on chlamydia control in Europe, 2015. *European Centre for Disease Prevention and Control*. http://doi.org/10.2900/667703

[68] Deniset, J. F., Cheung, P. K. M., Dibrov, E., Lee, K., Steigerwald, S., & Pierce, G. N. (2010). *Chlamydophila pneumoniae* Infection Leads to Smooth Muscle Cell Proliferation and Thickening in the Coronary Artery without Contributions from a Host Immune Response. *The American Journal of Pathology*, *176*(2), 1028–1037. http://doi.org/10.2353/ajpath.2010.090645

[69] European Centre for Disease Prevention and Control (2016). Guidance on Chlamydia control in Europe – 2015.

[70] *Final Recommendation Statement: Chlamydia and Gonorrhea: Screening*. U.S. Preventive Services Task Force. December 2016. https://www.uspreventiveservicestaskforce.org/Page/Document/RecommendationStatementFinal/chlamydia-and-gonorrhea-screening

[71] Lanjouw, E., Ouburg, S., de Vries, H. J., Stary, A., & Radcliffe, K. (2016). 2015 European guideline on the management of *Chlamydia trachomatis* infections. *International Journal of STD & AIDS*, *27*(5), 333–48. http://doi.org/10.1177/0956462415618837

[72] Gomes, J. P., Borrego, M. J., Atik, B., Santo, I., Azevedo, J., Brito de Sá, A., Nogueira, P., & Dean, D. (2006). Correlating *Chlamydia trachomatis* infectious load with urogenital ecological success and disease pathogenesis. *Microbes and Infection*, *8*, 16–26. http://doi.org/10.1016/j.micinf.2005.05.014

[73] Rizzo, A., Domenico, M., Carratelli, C. R., & Paolillo, R. (2012). The Role of *Chlamydia* and *Chlamydophila* Infections in Reactive Arthritis. *Internal Medicine*, *51*, 113–117. http://doi.org/10.2169/internalmedicine.51.6228

[74] Scholes, D., Stergachis, A., Heidrich, F. E., Andrilla, H., Holmes, K. K., & Stamm, W. E. (1996). Prevention Of Pelvic Inflammatory Disease By Screening For Cervical Chlamydial Infection. *The New England Journal of Medicine*, *334*(21), 1362–1366.

[75] Kamwendo, F., Forslin, L., Bodin, L., & Danielsson, D. (1996). Decreasing incidences of gonorrhea- and chlamydia-associated acute pelvic inflammatory disease: a 25-year study from an urban area of central Sweden. *Sexually Transmitted Diseases, 23*(5), 384-91.

[76] Suarez, J. P., Sanchez, L. R., Salazar, F. C., Saka, H. A., Tissera, A., Rivero, V. E., et. al (2017). *Chlamydia trachomatis* neither exerts deleterious effects on spermatozoa nor impairs male fertility. *Scientific Reports*, *7*(1126), 1–14. http://doi.org/10.1038/s41598-017-01262-w

[77] Geisler, W. M., Wang, C., Morrison, S. G., Black, C. M., Bandea, C. I., & Iii, E. W. H. (2008). The Natural History of Untreated *Chlamydia trachomatis* Infection in the Interval Between Screening and Returning for Treatment. *Sexually Transmitted Diseases*, *35*(2), 119–123. http://doi.org/10.1097/OLQ.0b013e318151497d

[78] Unemo, M., & Shafer, W. M. (2011). Antibiotic resistance in *Neisseria gonorrhoeae*: origin, evolution, and lessons learned for the future. *Annals of the New York Academy of Sciences*, *1230*, 1–15. http://doi.org/10.1111/j.1749-6632.2011.06215.x.

[79] Stary, G., & Stary, A. (2008). Lymphogranuloma venereum outbreak in Europe. *Journal of the German Society of Dermatology*, *6*(11), 935–939. http://doi.org/10.1111/j.1610-0387.2008.06742.x

[80] van Hal, S. J., Hillman, R., Stark, D. J., Harkness, J. L., & Marriott, D. (2007). Lymphogranuloma venereum: an emerging anorectal disease in Australia. *Medical Journal of Australia*, *187*(5), 309–310.

[81] Nieuwenhuis, R. F., Ossewaarde, J. M., & Go, H. M. (2004). Resurgence of Lymphogranuloma Venereum in Western Europe: An Outbreak of *Chlamydia trachomatis* Serovar L2 Proctitis in The Netherlands among Men Who Have Sex with Men. *Clinical Infectious Diseases*, *39*, 996–1003.

[82] de Vries, H. J. C., Zingoni, A., Kreuter, A., Moi, H., & White, J. A. (2015). 2013 European Guideline on the Management of Lymphogranuloma Venereum. *Journal of the European Academy of Dermatology and Venereology*, *29*(1), 1–16. http://doi.org/10.1111/jdv.12461

[83] Van der Bij, A. K., Spaargaren, J., Morre, S. A., Fennema, H. S. A., Mindel, A., Coutinho, R. A., & de Vries, H. J. C. (2006). Diagnostic and Clinical Implications of Anorectal Lymphogranuloma Venereum in Men Who Have Sex with Men: A Retrospective Case-Control Study. *Clinical Infectious Diseases*, *42*(2), 186–194. http://doi.org/10.1086/498904

[84] Llibre, J. M., Nielsen, H., Heras, E., Matas, L., Lugo, R., Clotet, B., & Sirera, G. (2010). Lymphogranuloma venereum proctocolitis : a silent endemic disease in men who have sex with men in industrialised countries. *European Journal of Clinical Microbiology & Infectious Diseases*, *29*, 917–925. http://doi.org/10.1007/s10096-010-0959-2

[85] Decker, C. F. (2016). Emerging sexually transmitted diseases: Hepatitis C, Lymphogranuloma venereum (LGV), and *Mycoplasma genitalium* infections. *Disease-a-Month*, *62*(8), 314–318. http://doi.org/10.1016/j.disamonth.2016.03.017

[86] Kropp, R. Y., & Wong, T. (2005). Emergence of lymphogranuloma venereum in Canada. *Canadian Medical Association Journal*, *172*(13). http://doi.org/10.1503/cmaj.050621

[87] Pinsky, L., Chiarilli, D. B., Klausner, J. D., Kull, R. M., O'Keefe, R., Heffer, C., & Seward Jr, S. L. (2012). Rates of asymptomatic nonurethral gonorrhea and chlamydia in a population of university men who have sex with men. *Journal of American College Health, 60*(6), 481–484. http://doi.org/10.1080/07448481.2012.690465.

[88] Park, J., Marcus, J. L., Pandori, M., Snell, A., Philip, S. S., & Bernstein, K. T. (2012). Sentinel Surveillance for Pharyngeal Chlamydia and Gonorrhea Among Men Who Have Sex With Men - San Francisco, 2010. *Sexually Transmitted Diseases*, *39*(6), 482–484. http://doi.org/10.1097/OLQ.0b013e3182495e2f

[89] van Rooijen, M. S., van der Loeff, M. F., Morré, S. A., van Dam, A. P., Speksnijder, A. G., & de Vries, H. J. (2015). Spontaneous pharyngeal *Chlamydia trachomatis* RNA clearance. A cross-sectional study followed by a cohort study of untreated STI clinic patients in Amsterdam, The Netherlands. *Sexually Transmitted Infection, 91*(3), 157–164. http://doi.org/10.1136/sextrans-2014-051633

[90] Hammerschlag, M. R. (1989). Chlamydial infections. *Journal of Pediatrics,114*, 727–734.

[91] Rastogi, S., Das, B., Salhan, S., & Mittal, A. (2003). Effect of treatment for *Chlamydia trachomatis* during pregnancy. *International Journal of Gynecology and Obstetrics*, *80*(2), 129–137. http://doi.org/10.1016/S0020-7292(02)00371-5

[92] WHO Guidelines for the treatment of *Chlamydia trachomatis*. (2016). *World Health Organization*.

[93] Tanaka, M., Matsumoto, T., Kumazawa, J., Nakayama, H., & Miyazaki, Y. (1991). *Chlamydia trachomatis* antigen can be detected in the urine sample of men with non-gonococcal urethritis. *Urological Research*, 235–236.

[94] Unemo, M., & Shafer, W. M. (2014). Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st Century: Past, evolution, and future. *Clinical Microbiology Reviews*, *27*(3), 587–613. http://doi.org/10.1128/CMR.00010-14

[95] Tavares, E., Fernandes, C., Borrego, M. J., Rodrigues, A., & Cardoso, J. (2012). Antibiotic resistance in *Neisseria gonorrhoeae* - Past, present and future. *Sociedade Portuguesa de Dermatologia E Venereologia*, *70*(4), 483–493.

[96] Morgan, M. K., & Decker, C. F. (2016). Gonorrhea. *Disease-a-Month*, *62*(8), 260–268. http://doi.org/10.1016/j.disamonth.2016.03.009

[97] WHO Guidelines for the Treatment of *Neisseria gonorrhoeae*. (2016). *World Health Organization*.

[98] Mayor, M. T., Roett, M. A., & Uduhiri, K. A. (2012). Diagnosis and Management of Gonococcal Infections. *American Family Physician*, *86*(10), 931–938.

[99] Sherrard, J., & Barlow, D. (1996). Gonorrhoea in men: clinical and diagnostic aspects. *Genitourinary Medicine*, *72*, 422–426.

[100] Detels, R., Green, A. M., Klausner, J. D., Katzenstein, D., Gaydos, C., Handsfield, H. H., et. Al (2011). The Incidence and Correlates of Symptomatic and Asymptomatic *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Infections in Selected Populations in Five Countries. *Sexually Transmitted Diseases*, *38*(6), 503–509.

[101] Parashar, J. R., & Schechtman, A. (2011). Returning traveler with painful penile mass. *Journal of Family Practice*, *60*(5), 285–287.

[102] Brunham, R. C., Gottlieb, S. L., & Paavonen, J. (2015). Pelvic Inflammatory Disease. *New England Journal of Medicine, 372*(21), 2039-2048. http://doi.org/ 10.1056/NEJMra1411426

[103] Liu, B., Roberts, C. L., Clarke M., Jorm, L., Hunt, J., & Ward, J. (2013). Chlamydia and gonorrhoea infections and the risk of adverse obstetric outcomes: a retrospective cohort study. Sexually Transmitted Infections*, 89*(8), 672-678. http://doi.org/10.1136/sextrans-2013-051118

[104] Coutanceau, B., Boujenah, J., & Poncelet, C. (2015). Gonococcal Chorioamnionitis with Antepartum Fetal Death In Utero. *Case Reports in Obstetrics and Gynecology*, *2015*, 1–2. http://doi.org/10.1155/2015/451247

[105] Bernstein, K. T., Marcus, J. L., Nieri, G., Philip, S. S., & Klausner, J. D. (2010). Rectal Gonorrhea and Chlamydia Reinfection Is Associated With Increased Risk of HIV Seroconversion. *Journal of Acquired Immune Deficiency Syndromes*, *53*(4), 537–543. http://doi.org/10.1097/QAI.0b013e3181c3ef29

[106] Ghys, P. D., Fransen, K., Diallo, M. O., Ettiègne-traoré, V., Coulibaly, I., Yeboué, K. M., et. al (1997). The associations between cervicovaginal HIV shedding, sexually transmitted diseases and immunosuppression in female sex workers in Abidjan, Côte d'Ivoire. *AIDS*, *11*(12), F85–F93.

[107] Cole, M. J., Spiteri, G., Jacobsson, S., Pitt, R., Grigorjev, V., & Unemo, M. (2015). Is the tide turning again for cephalosporin resistance in *Neisseria gonorrhoeae* in Europe? Results from the 2013 European surveillance. *BMC Infectious Diseases*, *15*(8), 321. http://doi.org/10.1186/s12879-015-1013-x

[108] Van de Laar, M., & Spiteri, G. (2012). Increasing trends of gonorrhoea and syphilis and the threat of drug-resistant gonorrhoea in Europe. *Eurosurveillance*, *17*(29), 1–3.

[109] Global action plan to control the spread and impact of antimicrobial resistance in *Neisseria gonorrhoeae*. (2012). *World Health Organization*.

[110] Petousis-harris, H., Paynter, J., Morgan, J., Saxton, P., Mcardle, B., Goodyear-smith, F., & Black, S. (2017). Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. *The Lancet*, *390*, 1–8. http://doi.org/10.1016/S0140-6736(17)31449-6

[111] Tully, J. G., Taylor-Robinson, D., Cole, R. M., & Rose, D. L. (1981). A newly discovered mycoplasma in the human urogenital tract. *The Lancet, 1*(8233), 1288-1291.

[112] Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D. et. al (1995). The Minimal Gene Complement of *Mycoplasma genitalium*. *Science, 270*(5235), 397–404. doi:10.1126/science.270.5235.397

[113] Perkins, M. J., & Decker, C. F. (2016). Non-gonococcal urethritis. *Disease-a-Month*, *62*(8), 274–279. http://doi.org/10.1016/j.disamonth.2016.03.011

[114] Razin, S., Yogev, D., & Naot, Y. (1998). Molecular Biology and Pathogenicity of Mycoplasmas. *Microbiology and Molecular Biology Reviews*, *62*(4), 1094–1156.

[115] Andersen, B., Sokolowski, I., & Olesen, F. (2007). *Mycoplasma genitalium*: prevalence and behavioural risk factors in the general population. *Sexually Transmitted Infections*, *83*, 237–241. http://doi.org/10.1136/sti.2006.022970

[116] Oakeshott, P., Aghaizu, A., Hay, P., Reid, F., Kerry, S., Atherton, H., et. al (2010). Is *Mycoplasma genitalium* in Women the "New Chlamydia?" A Community-Based Prospective Cohort Study. *Clinical Infectious Diseases*, *51*, 1160–1166. http://doi.org/10.1086/656739

[117] Falk, L., Fredlund, H., & Jensen, J. S. (2005). Signs and symptoms of urethritis and cervicitis among women with or without *Mycoplasma genitalium* or *Chlamydia trachomatis* infection. *Sexually Transmitted Infections*, *81*, 73–78. http://doi.org/10.1136/sti.2004.010439

[118] Manhart, L. E., Holmes, K. K., James, P., Houston, L. S., & Totten, P. A. (2007). *Mycoplasma genitalium* Among Young Adults in the United States: An Emerging Sexually Transmitted Infection. *American Journal of Public Health*, *97*(6), 1118–1125. http://doi.org/10.2105/AJPH.2005.074062

[119] Gesink, D. C., Mulvad, G., Montgomery-andersen, R., Poppel, U., Montgomery-andersen, S., Binzer, A., et. al (2012). *Mycoplasma genitalium* presence, resistance and epidemiology in Greenland. *International Journal of Circumpolar Health*, *71*(18203), 1–8.

[120] Kolarić, B., & Marijan, T. (2017). Assessing the Need for Routine Screening for *Mycoplasma genitalium* in the Low-risk Female Population: A Prevalence and Co-infection Study on Women from Croatia. *International Journal of Preventive Medicine*, *8*(51), 10–12. http://doi.org/10.4103/ijpvm.IJPVM

[121] Hooton, T. M., Roberts, M. C., Roberts, P. L., Holmes, K. K., Stamm, W. E., & Kenny, G. E. (1988). Prevalence of *Mycoplasma genitalium* determined by DNA probe in men with urethritis. *The Lancet, 1*(8580), 266-268

[122] Jensen, J. S., Hansen, H. T., & Lind, K. (1996). Isolation of *Mycoplasma genitalium* strains from the Male Urethra. *Journal of Clinical Microbiology*, *34*(2), 286–291.

[123] Bradshaw, C. S., Jensen, J. S., Tabrizi, S. N., Read, T. R. H., Garland, S. M., Hopkins, C. A., et. al (2006). Azithromycin Failure in *Mycoplasma genitalium* Urethritis. *Emerging Infectious Diseases*, *12*(7), 1149–1152. http://doi.org/10.3201/eid1207.051558

[124] Twin, J., Jensen, J. S., Bradshaw, C. S., Garland, S. M., Fairley, C. K., Min, L. Y., & Tabrizi, S. N. (2012). Transmission and Selection of Macrolide Resistant *Mycoplasma genitalium* Infections Detected by Rapid High Resolution Melt Analysis. *PLoS ONE*, *7*(4), 1–8. http://doi.org/10.1371/journal.pone.0035593

[125] Manhart, L. E., Broad, J. M., & Golden, M. R. (2011). *Mycoplasma genitalium*: Should We Treat and How? *Clinical Infectious Diseases*, *53*(6), S129-142. http://doi.org/10.1093/cid/cir702

[126] Bradshaw, C. S., Chen, M. Y., & Fairley, C. K. (2008). Persistence of *Mycoplasma genitalium* Following Azithromycin Therapy. *PLoS ONE*, *3*(11), 1–6. http://doi.org/10.1371/journal.pone.0003618

[127] Vandepitte, J., Weiss, H. A., Bukenya, J., Kyakuwa, N., Muller, E., Buvé, A., et. al (2014). Association between *Mycoplasma genitalium* infection and HIV acquisition among female sex workers in Uganda: evidence from a nested case – control study. *Sexually Transmitted Infections*, 6–11. http://doi.org/10.1136/sextrans-2013-051467

[128] Miles, R. J., Taylor, R. R., & Varsani, H. (1991). Oxygen uptake and H_2O_2 production by fermentative *Mycoplasma* spp. *Journal of Medical Microbiology*, *34*(4), 219–223. http://doi.org/10.1099/00222615-34-4-219

[129] Cohen, C. R., Nosek, M., Meier, A., Astete, S. G., Iverson-cabral, S., Mugo, N. R., et. al (2007). *Mycoplasma genitalium* Infection and Persistence in a Cohort of Female Sex Workers in Nairobi, Kenya. *Sexually Transmitted Diseases*, *34*(5), 274–279. http://doi.org/10.1097/01.olq.0000237860.61298.54

[130] Mena, L., Wang, X., Mroczkowski, T. F., & Martin, D. H. (2002). *Mycoplasma genitalium* Infections in Asymptomatic Men and Men with Urethritis Attending a Sexually Transmitted Diseases Clinic in New Orleans. *Clinical Infectious Diseases*, *35*(10), 1167–1173. http://doi.org/10.1086/343829

[131] Harp, D. F., & Chowdhury, I. (2011). Trichomoniasis: evaluation to execution. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *157*(1), 1–19. http://doi.org/10.1016/j.ejogrb.2011.02.024

[132] Gould, S. B., Woehle, C., Kusdian, G., Landan, G., Tachezy, J., Zimorski, V., et al. (2013). Deep sequencing of *Trichomonas vaginalis* during the early infection of vaginal epithelial cells and amoeboid transition. *International Journal for Parasitology,* *43*(9), 707-719. http://doi.org/10.1016/j.ijpara.2013.04.002

[133] Muñoz, C., Pérez, M., Orrego, P. R., Osorio, L., Gutiérrez, B., Sagua, H., et al. (2012). A protein phosphatase 1 gamma (PP1γ) of the human protozoan parasite *Trichomonas vaginalis* is involved in proliferation and cell attachment to the host cell. *International Journal for Parasitology, 42*(8), 715-727. http://doi.org/10.1016/j.ijpara.2012.03.012

[134] Edwards, T., Burke, P., Smalley, H., & Hobbs, G. (2016). *Trichomonas vaginalis*: Clinical relevance, pathogenicity and diagnosis. *Critical Reviews in Microbiology*, *42*(3), 406–417. http://doi.org/10.3109/1040841X.2014.958050

[135] Mielczarek, E., & Blaszkowska, J. (2016). *Trichomonas vaginalis*: pathogenicity and potential role in human reproductive failure. *Infection*, *44*(4), 447–458. http://doi.org/10.1007/s15010-015-0860-0

[136] Razin, S., Yogev, D., & Naot, Y. (1998). Molecular Biology and Pathogenicity of Mycoplasmas. *Microbiology and Molecular Biology Reviews*, *62*(4), 1094–1156.

[137] Zubacova Z., Cimburek Z., & Tachezy J. 2008. Comparative analysis of trichomonad genome sizes and karyotypes. *Molecular and Biochemical Parasitology*, *161*(1), 49–54. http://doi.org/ 10.1016/j.molbiopara.2008.06.004

[138] Huppert, J. S., Batteiger, B. E., Braslins, P., Feldman, J. A., Hobbs, M. M., Sankey, H. Z., et. al (2005). Use of an Immunochromatographic Assay for Rapid Detection of *Trichomonas vaginalis* in Vaginal Specimens. *Journal of Clinical Microbiology*, *43*(2), 684–687. http://doi.org/10.1128/JCM.43.2.684

[139] Akosile, W., & McDermott, B. M. (2017). Prevalence and clinical correlates of chlamydia infection in youth who use drugs and alcohol: a good opportunity for early intervention. *Australasian Psychiatry*, *25*(2), 146–149. http://doi.org/10.1177/1039856216689536

[140] Krashin, J. W., Koumans, E. H., Bradshaw-Sydnor, A. C., Braxton, J. R., Secor, W. E., Sawyer, M. K., et. al (2010). *Trichomonas vaginalis* Prevalence, Incidence, Risk Factors and Antibiotic-Resistance in an Adolescent Population. *Sexually Transmitted Diseases*, *37*(7), 440–444. http://doi.org/10.1097/OLQ.0b013e3181cfcd8c

[141] Petrin, D., Delgaty, K., Bhatt, R., & Garber, G. (1998). Clinical and Microbiological Aspects of *Trichomonas vaginalis*. *Clinical Microbiology Reviews*, *11*(2), 300–317.

[142] Miller, W. C., Hobbs, M. M., Schwebke, J. R., Leone, P. A., Swygard, H., Atashili, J., & Cohen, M. S. (2007). *Trichomonas vaginalis* Infection in Male Sexual Partners: Implications for Diagnosis, Treatment, and Prevention. *Clinical Infectious Diseases*, *44*(1), 13–22.

[143] Habib, F. S., Metwally, D. M., & Habib, K. S. (2004). Cryopreservation of *Trichomonas vaginalis*: a trial of using four different cryoprotectants. *Journal of the Egyptian Society of Parasitology, 34*(3), 931-940.

[144] Van Der Pol, B., Kraft, C. S., & Williams, J. A. (2006). Use of an Adaptation of a Commercially Available PCR Assay Aimed at Diagnosis of Chlamydia and Gonorrhea To Detect *Trichomonas vaginalis* in Urogenital Specimens. *Journal of Clinical Microbiology*, *44*(2), 366–373. http://doi.org/10.1128/JCM.44.2.366

[145] Poole, D. N., & Mcclelland, R. S. (2013). Global epidemiology of *Trichomonas vaginalis*. *Sexually Transmitted Infections*, *89*, 418–422. http://doi.org/10.1136/sextrans-2013-051075

[146] Zhang, Z. F., Graham, S., Yu, S. Z., Marshall, J., Zielezny, M., Chen, Y. X., et al. (1995). *Trichomonas vaginalis* and cervical cancer. A prospective study in China. *Annals of Epidemiology, 5*(4), 325-332

[147] Sutton, M., Sternberg, M., Koumans, E. H., Mcquillan, G., Berman, S., & Markowitz, L. (2007). The Prevalence of *Trichomonas vaginalis* Infection among Reproductive-Age Women in the United States, 2001-2004. *Clinical Infectious Diseases*, *45*, 1319–1326. http://doi.org/10.1086/522532

[148] Rein, M.F. (1990). Clinical Manifestations of Urogenital Trichomoniasis in Women. *Trichomonads Parasitic in Humans*, 225-234. https://doi.org/10.1007/978-1-4612-3224-7_11

[149] Fichorova, R. N., Buck, O. R., Yamamoto, H. S., Fashemi, T., Dawood, H. Y., Fashemi, B., et. al (2013). The Villain Team-Up or how *Trichomonas vaginalis* and bacterial vaginosis alter innate immunity in concert. *Sexually Transmitted Infections*, *89*, 460–466. http://doi.org/10.1136/sextrans-2013-051052

[150] Heine, P., & McGregor, J. A. (1993). *Trichomonas vaginalis*: a reemerging pathogen. *Clinical Obstetrics and Gynecology, 36*(1), 137-144.

[151] El-Shazly, A. M., El-Naggar, H. M., Soliman, M., El-Negeri, M., El-Nemr, H. E., Handousa, A.E., et al. (2001). A study on Trichomoniasis vaginalis and female infertility. *Journal of the Egyptian Society of Parasitology, 31*(2), 545-543.

[152] Swygard, H., Seña, A. C., Hobbs, M. M., & Cohen, M. S. (2004). Trichomoniasis: clinical manifestations, diagnosis and management. *Sexually Transmitted Infections*, *80*(2), 91–96. http://doi.org/10.1136/sti.2003.005124

[153] Laga, M., Manoka, A., Kivuvu, M., Malele, B., Tuliza, M., Nzila, N., et al. (1993). Non-ulcerative sexually transmitted diseases as risk factors for HIV-1 transmission in women: results from a cohort study. *AIDS, 7*(1), 95-102

[154] Silver, B. J., Guy, R. J., Kaldor, M., Jamil, M. S., & Rumbold, A. R. (2014). *Trichomonas vaginalis* as a Cause of Perinatal Morbidity: A Systematic Review and Meta-Analysis. *Sexually Transmitted Diseases*, *41*(6), 369–376. http://doi.org/10.1097/OLQ.0000000000000134

[155] Simhan, H. N., Caritis, S. N., Krohn, M. A., & Hillier, S. L. (2005). The vaginal inflammatory milieu and the risk of early premature preterm rupture of membranes. *American Journal of Obstetrics & Gynecology, 192*(1), 213-218. http://doi.org/10.1016/j.ajog.2004.07.021

[156] Smith, L. M., Wang, M., Zangwill, K., & Yeh, S. (2002). *Trichomonas vaginalis* Infection in a Premature Newborn. *Journal of Perinatology*, *22*, 502–503. http://doi.org/10.1038/sj.jp.7210714

[157] Iqbal, J., Al-rashed, J., & Kehinde, E. O. (2016). Detection of *Trichomonas vaginalis* in prostate tissue and serostatus in patients with asymptomatic benign prostatic hyperplasia. *BMC Infectious Diseases*, 1–7. http://doi.org/10.1186/s12879-016-1843-1

[158] Hobbs, M. M., & Seña, A. C. (2013). Modern diagnosis of *Trichomonas vaginalis infection*. *Sexually Transmitted Infections*, *89*(6), 434–438. http://doi.org/10.1136/sextrans-2013-051057

[159] Nye, M. B., Schwebke, J. R., & Body, B. A. (2009). Comparison of APTIMA *Trichomonas vaginalis* transcription-mediated amplification to wet mount microscopy, culture, and polymerase chain reaction for diagnosis of trichomoniasis in men and women. *American Journal of Obstetrics & Gynecology, 200*(2). http://doi.org/10.1016/j.ajog.2008.10.005

[160] Barbara, Š., Skvar, M., & Mati, M. (2015). Trichomoniasis : a brief review of diagnostic methods and our experience with real-time PCR for detecting infection. *Acta Dermatovenerol APA*, *24*(7), 7–10. http://doi.org/10.15570/actaapa.2015.3

[161] Kissinger, P. (2015). *Trichomonas vaginalis*: a review of epidemiologic, clinical and treatment issues. *BMC Infectious Diseases*, *15*(307), 1–8. http://doi.org/10.1186/s12879-015-1055-0

[162] Schmid, G., Narcisi, E., Mosure, D., Secor, W. E., Higgins, J., & Moreno, H. (2001). Prevalence of metronidazole-resistant *Trichomonas vaginalis* in a gynecology clinic. *Journal of Reproductive Medicine, 46*(6), 545-549

[163] Lan, J., Walboomers, J. M. M., Roosendaal, R., van Doornum, G. J. J., Maclaren, D. M., Meijer, C. J. L. M., & van den Brulel, A. J. C. (1993). Direct Detection and Genotyping of *Chlamydia trachomatis* in Cervical Scrapes by Using Polymerase Chain Reaction and Restriction Fragment Length Polymorphism Analysis. *Journal of Clinical Microbiology*, *31*(5), 1060–1065

[164] Sequencing chemistry. Available from: http://www.appliedbiosystems.com/absite/us/en/home/applications-technologies/dna-sequencing-fragment-analysis/overview-of-dna-sequencing/sequencing-chemistries.html

[165] Pinto, M., Borges, V., Antelo, M., Pinheiro, M., Nunes, A., Azevedo, J., et. al (2016). Genome-scale analysis of the non-cultivable *Treponema pallidum* reveals extensive within-patient genetic variation. *Nature Microbiology*, *2*, 1–11. http://doi.org/10.1038/nmicrobiol.2016.190

[166] Caldeira, A. R. S. V. (2017). *Propagation and molecular characterization of C. trachomatis strains isolated in Portugal*. Master's Thesis in Biomedical Sciences, specialty of Molecular Biology in

Tropical and International Medicine – Instituto de Higiene e Medicina Tropical – Universidade Nova de Lisboa, Lisbon. 81pp.

[167] Watson, E. J., Templeton, A., Russell, I., Paavonen, J., Mardh, P., Stary, A., et. al (2002). The accuracy and efficacy of screening tests for *Chlamydia trachomatis*: a systematic review. *Journal of Medical Microbiology*, *51*, 1021–1031

[168] Lanjouw, E., Ossewaarde, J. M., Stary, A., Boag, F., & van der Meijden, W. I. (2010). 2010 European guideline for the management of *Chlamydia trachomatis* infections. *International Journal of STD & AIDS*, *21*(11), 729–737. http://doi.org/10.1258/ijsa.2010.010302

[169] Chernesky, M., Jang, D., Chong, S., Sellors, J., & Mahony, J. (2003). Impact of urine collection order on the ability of assays to identify *Chlamydia trachomatis* infections in men. *Sexually Transmitted Diseases, 30*(4), 345–347.

[170] Parra-sánchez, M., Carlos, J., Bernal, S., Trinidad, M., Sivianes, N., Pérez, L., et. al (2012). Evaluation of the cobas 4800 CT/NG Test for detecting *Chlamydia trachomatis* and *Neisseria gonorrhoeae* DNA in urogenital swabs and urine specimens. *Diagnostic Microbiology and Infectious Disease*, *74*(4), 338–342. http://doi.org/10.1016/j.diagmicrobio.2012.08.004

[171] Van Der Pol, B., Liesenfeld, O., Williams, J. A., Taylor, S. N., Lillis, R. A., Body, B. A., et. al (2012). Performance of the cobas CT/NG Test Compared to the Aptima AC2 and Viper CTQ/GCQ Assays for Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *Journal of Clinical Microbiology*, *50*(7), 2244–2249. http://doi.org/10.1128/JCM.06481-11

[172] De Baetselier, I., Smet, H., Vuylsteke, B., & Crucitti, T. (2017). *Mycoplasma genitalium* and *Trichomonas vaginalis* detection in a cohort of men who have sex with men in Belgium: evaluation of the Diagenode S-DiaMGTV multiplex kit. *Sexually Transmitted Infections*, *93*, A53LP–A53.

[173] Bachmann, L. H., Johnson, R. E., Cheng, H., Markowitz, L., Papp, J. R., Palella, F. J., et. al (2010). Nucleic Acid Amplification Tests for Diagnosis of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* Rectal Infections. *Journal of Clinical Microbiology*, *48*(5), 1827–1832. http://doi.org/10.1128/JCM.02398-09

[174] Gorbach, P. M., Manhart, L. E., Hess, K. L., Stoner, B. P., Martin, D. H., & Holmes, K. K. (2009). Anal intercourse among young heterosexuals in three sexually transmitted disease clinics in the United States. *Sexually Transmitted Diseases, 36*, 193–198.

[175] Hoebe, C., van Liere, G., Niekamp, A. M., & Dukers-Muijrers, N. (2011). Many missed STI when only testing urogenitally without systematic anorectal and oropharyngeal screening in swingers and MSM. *Sexually Transmitted Infections*, *87*, 6–8. http://doi.org/10.1136/sextrans-2011-050109.40

[176] Peters, R. P. H., Mutsaers, J., Jansen, C. L., Morre, S. A., & van Leeuwen, A. P. (2011). Screening of Oropharynx and Anorectum Increases Prevalence of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Infection in Female STD Clinic Visitors. *Sexually Transmitted Diseases*, *38*(9), 783–787. http://doi.org/10.1097/OLQ.0b013e31821890e9

[177] Clinic-based testing for rectal and pharyngeal *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections by community-based organizations—five cities, United States, 2007 (2009). *Morbidity and Mortality Weekly Report – CDC*, *58*, 716 –719.

[178] Kent, C. K., Chaw, J. K., Wong, W., Liska, S., Gibson, S., Hubbard, G., et al (2005). Prevalence of rectal, urethral, and pharyngeal chlamydia and gonorrhea detected in 2 clinical settings among men who have sex with men: San Francisco, California, 2003. *Clinical Infectious Diseases, 41*(1), 67–74. http://doi.org/10.1086/430704

[179] Tilson, E. C., Sanchez, V., Ford, C. L., Smurzynski, M., Leone, P. A., Fox, K. K. et al. (2004). Barriers to asymptomatic screening and other STD services for adolescents and young adults: focus group discussions. *BMC Public Health, 4*, 1–8. http://doi.org/10.1186/1471-2458-4-21

[180] Paul, I. D., & Caul, E. O. (1990). Evaluation of three *Chlamydia trachomatis* immunoassays with an unbiased, noninvasive clinical sample. *Journal of Clinical Microbiology, 28*(2), 220–222.

[181] Geisler, W. M., Lensing, S. Y., Press, C. G., & Iii, E. W. H. (2013). Spontaneous Resolution of Genital *Chlamydia trachomatis* Infection in Women and Protection from Reinfection. *Journal of Infectious Diseases*, *207*, 1850–1856. http://doi.org/10.1093/infdis/jit094

[182] Schofield, M. J., Minichiello, V., Mishra, G. D., Plummer, D., & Savage, J. (2000). Sexually transmitted infections and use of sexual health services among young Australian women: Women's health Australia study. *International Journal of STD & AIDS, 11,* 313–323.

[183] Vall-Mayans, M., Caballero, E., & Sanz, B. (2009). The emergence of lymphogranuloma venereum in Europe. *Lancet*, *374*(9686), 356. https://doi.org/10.1016/S0140-6736(09)60993-4

[184] Getman, D., Jiang, A., O'Donnell, M., & Cohen, S. (2016). *Mycoplasma genitalium* Prevalence, Coinfection, and Macrolide Antibiotic Resistance Frequency in a Multicenter Clinical Study Cohort in the United States. *Journal of Clinical Microbiology*, *54*(9), 2278–2283. http://doi.org/10.1128/JCM.01053-16.Editor

[185] Mobley, V. L., Hobbs, M. M., Lau, K., Weinbaum, B. S., Getman, D. K., & Seña, A. C. (2012). *Mycoplasma genitalium* infection in women attending an STI clinic: diagnostic specimen type, co-infections, and predictors Victoria. *Sexually Transmitted Diseases*, *39*(9), 706–709. http://doi.org/10.1097/OLQ.0b013e318255de03.

[186] Gratrix, J., Plitt, S., Turnbull, L., Smyczek, P., Brandley, J., Scarrott, R., et. al (2017). Prevalence and antibiotic resistance of *Mycoplasma genitalium* among STI clinic attendees in Western Canada: a cross-sectional analysis. *BMJ Open*, *7*(7), e016300. http://doi.org/10.1136/bmjopen-2017-016300

[187] Meites, E., Llata, E., Braxton, J., Schwebke, J. R., Bernstein, K. T., Pathela, P., … Weinstock, H. S. (2015). *Trichomonas vaginalis* in Selected US Sexually Transmitted Disease Clinics: Testing, Screening, and Prevalence. *Sexually Transmitted Diseases*, *40*(11), 865–869. http://doi.org/10.1097/OLQ.0000000000000038.

[188] Helms, D. J., Mosure, D. J., Metcalf, C. A., Douglas, J. M. Jr., Malotte, C. K., Paul, S. M., et al. (2008). Risk factors for prevalent and incident *Trichomonas vaginalis* among women attending three sexually transmitted disease clinics. *Sexually Transmitted Diseases, 35*(5), 484–488. http://doi.org/10.1097/OLQ.0b013e3181644b9c

[189] Johnson, H. L., Ghanem, K. G., Zenilman, J. M., & Erbelding, E. J. (2011). Sexually transmitted infections and adverse pregnancy outcomes among women attending inner city public sexually

transmitted diseases clinics. *Sexually Transmitted Diseases, 38*(3), 167–171. http://doi.org/10.1097/OLQ.0b013e3181f2e85f

[190] Instituto Nacional de Saúde Doutor Ricardo Jorge, IP (2016). Infeção VIH/SIDA: a situação em Portugal a 31 de dezembro de 2015 / Unidade de Referência e Vigilância Epidemiológica, Departamento de Doenças Infeciosas.

[191] Barbeyrac, B., & Bébéar, C. (2009). Genital *Chlamydia trachomatis* infections. *Clinical Microbiology and Infection*, *15*(1), 4–10. http://doi.org/10.1111/j.1469-0691.2008.02647.x

[192] Borrego, M. J., Gomes, J. P., Lefebvre, J. F., Eb, F., Orfila, J., & Catry, M. A. (1997). Genotyping of Portuguese *Chlamydia trachomatis* urogenital isolates. *Genitourinary Medicine*, *73*, 561–563.

[193] Brasiliense, D. M., Borges, N., Augusto, W., & Ferreira, S. (2016). Genotyping and prevalence of *Chlamydia trachomatis* infection among women in Belém, Pará, northern Brazil. *The Journal of Infection in Developing Countries*, *10*(2), 9–14. http://doi.org/10.3855/jidc.6474

[194] Beni, B. T., Motamedi, H., & Ardakani, M. R. (2017). Genotyping of the prevalent *Chlamydia trachomatis* strains involved in cervical infections in women in Ahvaz, Iran. *Journal of Medical Microbiology*, *59*, 1023–1028. http://doi.org/10.1099/jmm.0.016717-0

[195] Haro-cruz, M. J., Deleón-rodriguez, I., Escobedo-guerra, M. R., López-hurtado, M., Arteaga-troncoso, G., Ortiz-ibarra, F. J., & Guerra-infante, F. M. (2011). Genotyping of *Chlamydia trachomatis* from endocervical specimens of infertile Mexican women. *Enfermedades Infecciosas Y Microbiología Clínica*, *29*(2), 102–108. http://doi.org/10.1016/j.eimc.2010.08.014

[196] Andreasen, A. A., Burton, M. J., Holland, M. J., Polley, S., Faal, N., Mabey, D. C., & Bailey, R.L. (2008). *Chlamydia trachomatis ompA* variants in trachoma: what do they tell us. *PLOS Neglected Tropical Diseases, 2*(9), e306. http://doi.org/10.1371/journal.pntd.0000306

[197] Millman, K., Black, C.M., Johnson, R.E., Stamm, W.E., Jones, R.B., Hook, E.W., et al. (2004). Population-based genetic and evolutionary analysis of *Chlamydia trachomatis* urogenital strain variation in the United States. *Journal of Bacteriology, 186*(8), 2457–2465. http://doi.org/ 10.1128/JB.186.8.2457-2465.2004

[198] Brunham, R. C., Yang, C., Maclean, I., Kimani, J., Maitha, G., & Plummer, F. (1994). *Chlamydia trachomatis* from individuals in a sexually transmitted disease core group exhibit frequent sequence variation in the major outer membrane protein (*omp1*) gene. *Journal of Clinical Investigation, 94*(1), 458–463. http://doi.org/10.1172/JCI117347

[199] Brunelle, B. W., & Sensabaugh, G. F. (2006). The ompA Gene in *Chlamydia trachomatis* Differs in Phylogeny and Rate of Evolution from Other Regions of the Genome. *Infection and Immunity*, *74*(1), 578–585. http://doi.org/10.1128/IAI.74.1.578

[200] Mabey, D., & Peeling, R. W. (2002). Lymphogranuloma venereum. *Sexually Transmitted Infections*, *78*, 90–92

[201] Barnes, R. C., Rompalo, A. M., & Stamm, W. E. (1987). Comparison of *Chlamydia trachomatis* Serovars Causing Rectal and Cervical Infections, *The Journal of Infectious Diseases*, 156(6), 953–958. https://doi.org/10.1093/infdis/156.6.953

[202] Hsu, M. C., Tsai, P. Y., Chen, K. T., Li, L. H., Chiang, C. C., Tsai, J. J. et al (2006). Genotyping of *Chlamydia trachomatis* from clinical specimens in Taiwan. *Journal of Medical Microbiology, 55*, 301–308. https://doi.org/10.1099/jmm.0.46262-0

[203] Abdelsamed, H., Peters, J., & Byrne, G. I. (2014). Genetic variation in *Chlamydia trachomatis* and their hosts: impact on disease severity and tissue tropism. *Future Microbiology*, *8*(9), 1129–1146. http://doi.org/10.2217/fmb.13.80.Genetic

[204] Bax, C. J., Quint, K. D., Peters, R. P. H., Ouburg, S., Oostvogel, P. M., Mutsaers, J. A. E. M. et al. (2011). Analyses of multiple-site and concurrent *Chlamydia trachomatis* serovar infections, and serovar tissue tropism for urogenital versus rectal specimens in male and female patients. *Sexually Transmitted Infections, 87*, 503–507. http://doi.org/10.1136/sti.2010.048173

[205] Jurstrand, M., Falk, L., Fredlund, H., Lindberg, M., Olcén, P., Andersson, S. et al. (2001). Characterization of *Chlamydia trachomatis omp1* genotypes among sexually transmitted disease patients in Sweden. *Journal of Clinical Microbiology, 39*(11), 3915–3919. https://doi.org/10.1128/JCM.39.11.3915-3919.2001

[206] Lister, N. A., Tabrizi, S. N., Fairley, C. K., Smith, A., Janssen, P. H., & Garland, S. (2004). Variability of the *Chlamydia trachomatis omp1* gene detected in samples from men tested in male-only saunas in Melbourne, Australia. *Journal of Clinical Microbiology, 42*(6), 2596–2601. https://doi.org/10.1128/JCM.42.6.2596-2601.2004

[207] Feher, V. A., Randall, A., Baldi, P., Bush, R. M., de la Maza, L. M., & Amaro, R. E. (2013). A 3-Dimensional Trimeric β-Barrel Model for *Chlamydia* MOMP Contains Conserved and Novel Elements of Gram-Negative Bacterial Porins. *PLoS ONE*, *8*(7), e68934. http://doi.org/10.1371/journal.pone.0068934

[208] Feener, E. P., Shen, W. C., & Ryser, H. J. (1990). Cleavage of disulfide bonds in endocytosed macromolecules. A processing not associated with lysosomes or endosomes. *Journal of Biological Chemistry*, *265*(31), 18780–18785

[209] Belland, R. J., Scidmore, M. A., Crane, D. D., Hogan, D. M., Whitmire, W., McClarty, G., et al. (2001). *Chlamydia trachomatis* cytotoxicity associated with complete and partial cytotoxin genes. *Proceedings of the National Academy of Sciences of the United States of America*, *98*(24), 13984–13989. http://doi.org/10.1073/pnas.241377698

[210] Bothe, M., Dutow, P., Pich, A., Genth, H., & Klos, A. (2015). DXD Motif-Dependent and -Independent Effects of the *Chlamydia trachomatis* Cytotoxin CT166. Toxins, *7*, 621–637.

[211] Wu, X., Lei, L., Gong, S., Chen, D., Flores, R., & Zhong, G. (2011). The chlamydial periplasmic stress response serine protease cHtrA is secreted into host cell cytosol. *BMC Microbiology*, *11*, 87. http://doi.org/10.1186/1471-2180-11-87

[212] Hower, S., Wolf, K., & Fields, K. A. (2009). Evidence that CT694 is a novel *Chlamydia trachomatis* T3S substrate capable of functioning during invasion or early cycle development. *Molecular Microbiology, 72*(6), 1423–1437. http://doi.org/10.1111/j.1365-2958.2009.06732.x

[213] Verma, A., & Maurelli, A. T. (2003). Identification of two eukaryote-like serine/threonine kinases encoded by *Chlamydia trachomatis* serovar L2 and characterization of interacting partners of Pkn1. *Infection and Immunity, 71*(10), 5772–84.

Annexes

Annex 1 – Oligonucleotides for nested-PCR.

Name	Sequence (5’ → 3’)
NLO	ATGAAAAAACTCTTGAAATCG
NRO	CTCAACTGTAACTGCGTATTT
PCTM3	TCCTTGCAAGCTCTGCCTGTGGGGAATCCT
SERO2A	TTTCTAGATTTCATCTTGTT

Annex 2 – Oligonucleotides for sequencing reaction.

Name	Sequence (5’ → 3’)
OMPA-1	TTATGATCGACGGAATTCT
SERO2A	TTTCTAGATTTCATCTTGTT

Annex 3 – Homopolymeric tracts [poly (N)] selected for the study.

Potential affected gene	Location of poly (N)	Poly (N) type *
CT042/glgX (glycogen hydrolase (debranching))	Possible regulatory region	G/C
CT166 (cytotoxin)	Coding region	G/C
CT172 (hypothetical protein)	Possible regulatory region	G/C
CT259 (PP2C phosphatase)	Possible regulatory region	G/C
CT326 (hypothetical protein)	Coding region	G/C
CT445/gltX (glutamyl-tRNA synthase)	Possible regulatory region	A/T
CT541/mip (peptidyl-prolyl cis-trans isomerase)	Possible regulatory region	A/T
CT561 (type III secretion system protein)	Possible regulatory region	A/T
CT605 (hypothetical protein)	Possible regulatory region	A/T
CT694 (type III secretion system protein)	Possible regulatory region	A/T
CT823/htrA (DO serine protease)	Possible regulatory region	A/T
CT871/pmpG (polymorphic membrane protein G)	Coding region	G/C

* The two hypotheses refer to the leading or lagging strand.

Annex 4 – Clinical samples for homopolymeric tracts study (samples marked with * are from Hadfield, J. et al., 2017 [48]).

Name	Anatomical Site	Genotype	Clade
CS752_14	Genital	B	T2
CS973_13	Genital	Da	T1
CHLC274	Genital	Da	T1
CS695_15	Rectal	Da	T1
CS301_14	Genital	E	T1
CS732_14	Genital	E	T1
CS746_14	Genital	E	T1
293230_16	Genital	E	T1
CS313_13	Genital	F	T1
CS189_15	Genital	F	T1
CHLC139	Rectal	G	T2
CHLC151	Genital	G	T2
CHLC179	Genital	G	T2
CHLC191	Oropharyngeal	G	T2
CHLC207	Rectal	G	T2
CS801_13	Genital	J	T2
CHLC57	Genital	J	T2
CHLC156	Rectal	J	T2
CS648_15	Rectal	L2	LGV
CS901_15	Rectal	L2	LGV
CHLC228	Rectal	L2b	LGV
CHLC277	Rectal	L2b	LGV
CHLC194	Genital	J	T2
CHLC243	Genital	E	T1
CHLC231	Rectal	L2b	LGV
CHLC214	Rectal	Da	T1
CHLC238	Rectal	Da	T1
CHLC200	Genital	E	T1
CHLC6/14	Rectal	E	T1
APDES163/16	Rectal	F	T1

APDES181AR/16	Rectal	E	T1
APDES188/16	Rectal	J	T2
CHLC108/15	Rectal	Da	T1
CHLC134/15	Rectal	L2	LGV
CHLC150/15	Rectal	L2	LGV
CHLC167/15	Rectal	G	T2
CHLC171/15	Rectal	L2	LGV
CHLC175/15	Rectal	Da	T1
CHLC224/16	Rectal	L2	LGV
CHLC229/16	Rectal	L2	LGV
CHLC232/16	Rectal	L2	LGV
CHLC13/14	Conjunctival	F	T1
CHLC23/14	Conjunctival	Da	T1
CHLC26/14	Conjunctival	Da	T1
CHLC56/14	Conjunctival	E	T1
CHLC62/14	Conjunctival	E	T1
CHLC178/15	Conjunctival	E	T1
CHLC271/16	Conjunctival	F	T1
CHLC313/16	Conjunctival	Da	T1
CS135/12	Rectal	G	T2
CHLC206/16	Oropharyngeal	G	T2
CHLC215/16	Oropharyngeal	G	T2
CHLC227/16	Oropharyngeal	G	T2
CHLC296/16	Oropharyngeal	G	T2
APDES181OR/16	Oropharyngeal	E	T1
CHLC314/16	Oropharyngeal	Da	T1
352445/17	Oropharyngeal	E	T1
150C/15	Genital	F	T1
S86/96	Genital	D	T2
150C/01	Genital	F	T1
1200C/06	Genital	Ia	T2
CS983/07	Genital	F	T1
CS1155/07	Genital	F	T1
CS1304/07	Genital	Ia	T2

CS32/07	Genital	F	T1
CS48/07	Genital	F	T1
CS503/07	Genital	F	T1
CS502/07	Genital	F	T1
CS579/08	Genital	Ia	T2
CS716/08	Genital	Ia	T2
285C/08	Genital	Ia	T2
687C/08	Genital	Ia	T2
CS86/09	Genital	H	T2
CS578/11	Genital	K	T2
184C/14	Genital	K	T2
CHLC266/16	Genital	K	T2
CS275/14	Genital	K	T2
634C/07	Genital	Ja	T1
CS278/11	Genital	Ja	T1
CHLC128/15	Genital	Ja	T1
CS57/13	Genital	Ja	T1
CS638/11	Genital	H	T2
CS132/13	Genital	H	T2
68C/11	Genital	H	T2
CS13/09	Genital	D	T2
CS521/09	Genital	D	T2
CS1290/10	Genital	D	T2
CS208/13	Genital	D	T2
CHLC354/16	Oropharyngeal	J	T2
CHLC325/16	Oropharyngeal	J	T2
CHLC225/16	Rectal	J	T2
CS382/11	Genital	E	T1
CS434/06	Genital	E	T1
CS933/07	Genital	E	T1
460C/10	Genital	E	T1
CHLC24/14	Genital	E	T1
D_HonLC4*	Unknown	D	T1
D_STN101*	Unknown	D	T2

D_STN113*	Unknown	D	T2
D_UK466322*	Genital	D	T2
D_UK663610*	Genital	D	T1
D_UK750364*	Genital	D	T1
D_UK750376*	Genital	D	T2
D_UK750523*	Genital	D	T2
D_UK912432*	Genital	D	T2
E_It246*	Genital	E	T1
E_It363*	Genital	E	T1
E_It769*	Genital	E	T1
E_It807*	Unknown	E	T1
E_STN10*	Unknown	E	T1
E_STN11*	Unknown	E	T1
E_STN115*	Unknown	E	T1
E_STN119*	Unknown	E	T1
E_STN12*	Unknown	E	T1
E_STN2*	Unknown	E	T1
E_STN47*	Unknown	E	T1
E_STN68*	Unknown	E	T1
E_STN92*	Unknown	E	T1
E_UK220880*	Genital	E	T1
E_UK34334*	Genital	E	T1
E_UK466129*	Genital	E	T1
E_UK466546*	Genital	E	T1
E_UK582206*	Genital	E	T1
E_UK582260*	Genital	E	T1
E_UK582263*	Genital	E	T1
E_UK583638*	Genital	E	T1
E_UK584031*	Genital	E	T1
E_UK663813*	Genital	E	T1
E_UK663924*	Genital	E	T1
E_UK663968*	Genital	E	T1
E_UK664394*	Genital	E	T1
E_UK769748*	Genital	E	T1

E_UK769852*	Genital	E	T1
E_UK912889*	Genital	E	T1
E_UK913723*	Genital	E	T1
E_UK913953*	Genital	E	T1
F_AddT9*	Unknown	F	T1
F_HonMPB36*	Genital	F	T1
F_It686*	Genital	F	T1
F_STN110*	Unknown	F	T1
F_STN15*	Unknown	F	T1
F_STN22*	Unknown	F	T1
F_UK220521*	Genital	F	T1
F_UK35155*	Genital	F	T1
F_UK465966*	Genital	F	T1
F_UK466273*	Genital	F	T1
F_UK583012*	Genital	F	T1
F_UK583072*	Genital	F	T1
F_UK583468*	Genital	F	T1
F_UK584026*	Genital	F	T1
F_UK663442*	Genital	F	T1
F_UK770010*	Genital	F	T1
G_UK221409*	Unknown	G	T2
G_UK582500*	Genital	G	T2
G_UK750369*	Genital	G	T2
G_UK913362*	Genital	G	T2
I_UK913341*	Genital	I	T2
J_UK35672*	Genital	J	T2
J_UK583546*	Genital	J	T2
J_UK583676*	Genital	J	T2
J_UK913454*	Genital	J	T2
K_UK582774*	Genital	K	T2
K_UK583237*	Genital	K	T2
K_UK663060*	Genital	K	T2
K_UK663124*	Genital	K	T2
K_Ur769079*	Genital	K	T2

L1_Ur583800*	Genital	L1	LGV

Annex 5 – Oligonucleotides for amplicon-based NGS.

Name	Oligonucleotide (5' → 3')
polyCT042	TGGCTCCTAGAGGTAAAGGGATAG
	CTGCGTGTTAGACATGAAGTATGC
polyCT166	CGAAGTTTATTACACAGCCAGTGA
	GTCCCGCTTCCGAATTTTTATCT
polyCT172	TCTCAACCAAATTCGGTAGAGGT
	GCTCCGGCTATTTTGTTTAGG
polyCT259	ATTCCGTCTGCAATGGCAATGA
	AAGGGAGGCAACTTTTGATACCC
polyCT326	TTCGCAGCAGAATCAAGACTCA
	AGGGGATTGGGTCGTTTAGAAG
polyCT445	CATTCTTGGCGACTTTAACAAAAC
	CTACACGCATTGGTGCTATGAAAG
polyCT541	GAACCGCCTCCGTTATCACATC
	TGCCGATTCAATTAAAAGAACTGG
polyCT561	TATTTTCGAGAGCGCTTAGAACAC
	ACCGCAGTATAGGCGTCTGG
polyCT605	GCTATCATCCTGCCCATACTCTC
	GCTGCAAATAAGCCTTTCTGAATA
polyCT694	TCAAAGCTAAATATCTGCGTATGC
	CCTCCGCCGAAGCAATAACTTTTA
polyCT823	ATGCTGCATATCTGCTTGTTAT
	CAATAGCCTGGTTCCCTGTTTTAG
polyCT871	TGCAAACGTCTTTCCATAAGTTCT
	GTCCGTATGTTCTCGAAAGTCAAC

Forward oligonucleotides are represented in grey, while reverse oligonucleotides are represented in white.

Annex 6 – Adapters sequence.

Overhang	Sequence (5' → 3')
Forward	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
Reverse	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

Annex 7 – Annealing temperatures for each primer used.

Primer	Annealing temperature (ºC)
polyCT042	54
polyCT166	54
polyCT172	54
polyCT259	54
polyCT326	54
polyCT445	54
polyCT541	55
polyCT561	55
polyCT605	54
polyCT694	55
polyCT823	55
polyCT871	54

Annex 8 – Distribution of *ompA*-genotypes per anatomical site of infection.

***ompA*-genotype**	**Anatomical site**			
	Anorectum	**Conjunctiva**	**Endocervix/Urethra**	**Oropharynx**
B	0	0	11	0
C	0	0	3	0
D	27	4	366	4
E	7	4	845	6
F	3	2	423	0
G	20	0	260	6
H	0	0	76	0
I	0	0	161	0
J	11	0	152	2
K	0	0	49	0
L1	0	0	1	0
L2	77	0	22	0
Mixed	1	0	35	0

Annex 9 – Distribution of *ompA*-genotypes per gender.

ompA-genotype	Gender	
	Female	**Male**
B	5	6
C	2	1
D	175	217
E	482	352
F	268	152
G	113	163
H	58	14
I	85	75
J	75	87
K	28	19
L1	1	0
L2	9	90
Mixed	27	9

Annex 10 – Distribution of *ompA*-genotypes per age.

***ompA*-genotype**	**Age groups (years)**				
	12 - 19	**20 - 24**	**25 - 34**	**35 - 44**	**45+**
B	0	2	5	2	1
C	1	0	2	0	0
D	58	97	146	49	19
E	143	227	275	96	35
F	76	125	140	38	10
G	28	81	108	35	13
H	10	13	26	12	3
I	37	42	57	13	6
J	20	45	54	18	13
K	5	10	22	5	1
L1	0	0	1	0	0
L2	2	6	30	41	19
Mixed	5	12	10	2	4

Annex 11 – Distribution of *ompA*-genotypes per year.

Year	*ompA*-genotype												
	B	C	D	E	F	G	H	I	J	K	L1	L2	Mixed
1990	0	0	3	0	0	0	0	0	0	0	0	0	0
1991	0	0	2	4	1	0	1	0	0	0	0	0	0
1992	0	0	4	12	1	1	2	0	1	0	0	0	0
1993	0	0	3	14	7	1	2	1	1	2	0	0	2
1994	1	0	2	18	4	8	5	2	2	0	1	0	3
1995	0	0	9	39	9	4	11	1	2	0	0	1	3
1996	1	0	5	28	7	4	14	3	3	0	0	2	1
1997	0	0	3	18	8	6	2	1	4	0	0	0	3
1998	0	0	1	11	0	1	0	1	0	2	0	0	0
1999	0	0	8	20	16	7	8	0	3	4	0	0	1
2000	1	0	4	19	9	2	0	3	2	2	0	0	0
2001	0	0	4	11	10	4	3	1	1	1	0	1	0
2002	0	0	9	16	10	8	1	1	3	0	0	0	1
2003	0	3	4	33	6	11	1	7	5	0	0	0	3
2004	0	0	14	41	11	12	0	6	6	1	0	0	3
2005	0	0	20	50	15	17	2	6	13	2	0	0	4
2006	3	0	25	76	32	14	3	17	5	1	0	0	1
2007	0	0	17	64	37	24	4	8	14	1	0	7	2
2008	1	0	17	39	19	9	0	10	7	4	0	4	2
2009	0	0	32	40	21	17	2	16	11	1	0	2	0
2010	0	0	19	28	28	18	4	4	15	0	0	6	1
2011	0	0	9	38	24	13	2	12	6	5	0	1	0
2012	1	0	16	28	14	10	0	6	9	2	0	0	0
2013	1	0	30	28	24	9	1	10	8	4	0	2	3
2014	1	0	23	46	19	12	1	11	4	5	0	2	1
2015	0	0	42	45	33	21	1	12	8	2	0	7	2
2016	0	0	49	64	49	40	6	12	20	10	0	35	0
2017	1	0	28	32	14	13	0	10	12	0	0	29	0

Annex 12 – Proportion of *ompA* prototype and variant strains among INSA's *C. trachomatis* collection.

***ompA*-genotype**	**Type of strain**	
	Prototype	**Variant**
B	7	3
C	0	3
D	92	279
E	694	22
F	361	9
G	37	217
H	5	41
I	0	152
J	84	71
K	38	1
L	69	26

Annex 13 – Intra-patient variability and profile of the homopolymeric tracts affecting **a)** CT166 **b)** CT042 **c)** CT694 **d)** CT561 **e)** CT605 **f)** CT541 **g)** CT172 **h)** CT445 **i)** CT871 **j)** CT326 **k)** CT823 **l)** CT259 in *C. trachomatis* strains, according to clade.

a) CT166

	Intra-patient variability		Dominant profile		
	Yes	**No**	**ON**	**OFF**	**Ocular-like**
T1 (n=60)	28	32	26	31	3
T2 (n=27)	12	15	5	8	14

b) CT042

	Intra-patient variability		Dominant profile	
	Yes	**No**	**Poly (6G)**	**GAGGGG**
T1 (n=74)	1	73	74	0
T2 (n=48)	2	46	11	27
LGV (n=8)	1	7	7	0

c) CT694

	Intra-patient variability		Dominant profile		
	Yes	**No**	**Poly (8T)**	**Poly (9T)**	**Poly (10T)**
T1 (n=72)	9	63	1	71	0
T2 (n=52)	6	46	9	38	5
LGV (n=9)	0	9	9	0	0

d) CT561

	Intra-patient variability		Dominant profile		
	Yes	**No**	**Poly (8A)**	**Poly (9A)**	**Poly (11A)**
T1 (n=65)	53	12	0	0	60
T2 (n=37)	4	33	12	25	0
LGV (n=7)	1	6	7	0	0

e) CT605

	Intra-patient variability		Dominant profile		
	Yes	**No**	**Poly (7T)**	**Poly (8T)**	**Poly (10T)**
T1 (n=86)	55	31	0	0	83
T2 (n=55)	3	52	0	48	0
LGV (n=10)	1	9	10	0	0

f) CT541

	Intra-patient variability		Dominant profile	
	Yes	**No**	**Poly (9A)**	**Poly (10A)**
T1 (n=87)	3	84	87	0
T2 (n=59)	25	34	30	29
LGV (n=10)	1	9	10	0

g) CT172

	Intra-patient variability		Dominant profile			
	Yes	**No**	**Poly (6C)**	**Poly (8C)**	**Poly (9C)**	**Poly (10C)**
T1 (n=80)	10	70	52	26	2	0
T2 (n=47)	27	20	1	5	20	13
LGV (n=10)	1	9	0	9	0	1

h) CT445

	Intra-patient variability		Dominant profile		
	Yes	**No**	**Poly (8A)**	**Poly (9A)**	**Poly (12A)**
T1 (n=55)	47	8	0	5	40
T2 (n=35)	11	24	0	30	0
LGV (n=8)	0	8	8	0	0

i) CT871

	Intra-patient variability		Dominant profile	
	Yes	**No**	**Poly (8G)**	**Poly (9G)**
T1 (n=80)	49	31	0	80
T2 (n=55)	40	15	0	55
LGV (n=10)	1	9	10	0

j) CT326

	Intra-patient variability		Dominant profile		
	Yes	**No**	**Poly (8C)**	**Poly (9C)**	**Poly (10C)**
T1 (n=85)	17	68	31	51	3
T2 (n=51)	3	48	45	2	0
LGV (n=8)	8	0	0	0	8

k) CT823

	Intra-patient variability		Dominant profile		
	Yes	**No**	**Poly (8A)**	**Poly (9A)**	**Poly (10A)**
T1 (n=64)	1	63	64	0	0
T2 (n=35)	6	29	2	32	1
LGV (n=8)	8	0	0	1	7

l) CT259

	Intra-patient variability		Dominant profile	
	Yes	**No**	**Poly (10C)**	**Poly (11C)**
T1 (n=54)	53	1	11	34
T2 (n=24)	13	11	1	6